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CONTENTS

	Page
THE CHEMISTRY OF THE DEGRADATION OF PROTEIN NITROGEN. *R. A. McCance*	1
CONTRACTURES OF SKELETAL MUSCLE. *Herbert S. Gasser*	35
THE ENZYMATIC SYNTHESIS OF PROTEIN. *Hardolph Wasteneys and Henry Borsook*	110
THE ERRORS OF BIOLOGICAL ASSAY. *J. H. Burn*	146

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H. M. EVANS: The Anterior Hypophysis
J. H. BURN: The Errors of Biological Assay
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S. W. BRITTON: Adrenal Insufficiency
BALDUIN LUCKÉ: Cellular Osmosis.
DAVID RAPPORT: The Interconversion of Foodstuffs
A. B. HASTINGS: Comparative Effects of Chlorides, Bromides and Iodides on Animals
CARL F. CORI: Carbohydrate Metabolism

PHYSIOLOGICAL REVIEWS

VOL. X JANUARY, 1930 No. 1

THE CHEMISTRY OF THE DEGRADATION OF PROTEIN NITROGEN

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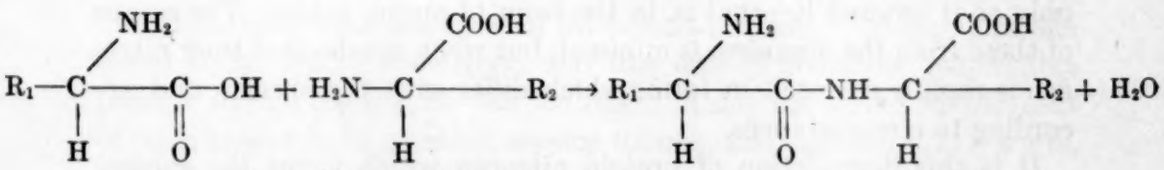
The old hypotheses of Voit and Pflüger that the protein molecule underwent metabolism as a whole are now only of historical interest so far as general metabolism is concerned. Such theories may still be of importance in questions of immunity and anaphylaxis, but these problems lie outside the scope of this review. For the last 30 or 40 years therefore those studying the intermediate metabolism of proteins have concentrated first upon their hydrolysis, and secondly upon the further metabolism of their constituent amino acids, of which some 22 are known.

While the actual structure of no single protein is known, the general principle underlying the construction of all of them is thought to be the same, i.e., the linking together of numbers of α-amino acids by the elimination of water between a carboxy and amino group. (Vickery and Osborne).

Thus:



Physiologically animals obtain all their protein from their food, but it cannot be regarded as entering the organism till it has left the lumen of the gut. The tissues, therefore, are protected from direct contact with "foreign" proteins by an organized series of ferments and a guardian epithelium, which together ensure that only the products of complete or practically complete hydrolysis enter the general circula-

tion. The allergic disturbances which follow the eating of specific proteins by certain people may be examples of the failure of this mechanism. (Mills et al, Van Alstyne and Grant, Wells.) Protozoa which engulf particles of protein are only an apparent exception to this, for these creatures submit the particles to complete digestion in an intracellular cavity (analogous to the alimentary tract), and only allow the products of digestion to reach the true interior of the cell. Protein material, therefore, is taken up by body cells mainly in the form of amino acids, put probably to some extent also in the form of simple peptides.

One of three fates awaits each amino acid which penetrates the membrane of a living cell. The first is condensation with a number of its fellows into one of the proteins characteristic of that cell. This is the converse of digestion. The two processes, condensation and hydrolysis, together form a complete system for the transport and assimilation of proteins, complete hydrolysis being the only path to specific synthesis. Within the cell these two types of reaction take place side by side, and which predominates (for the equilibrium is a dynamic one), depends upon the conditions prevailing at the time.

The second fate awaiting amino-acids is utilization for some specific purpose. The number of reactions in this class is large, and each one naturally a law unto itself. Included are cases of protective synthesis, formation of hormones, bile salts, catalysts, purines and pigments, and other reactions not yet understood.

The third type is deamination, and oxidation of the residue, which is usually complete but may abnormally be incomplete.

Leaving aside the action of the true secretory cells, such as those of the mammary gland, protein material probably leaves the healthy cell only as it entered it—that is, in the form of amino acids. The escape of these from the organism is minimal, but when catabolized their nitrogen is rapidly excreted in forms which differ with the species, and according to circumstances.

It is this degradation of protein nitrogen which forms the subject of the present review, but the general principles which are well understood need not detain us. Thus all are agreed that mammals excrete 80 to 90 per cent of the nitrogen of the protein they metabolize as urea and ammonia, and that the latter forms normally only a small percentage of the total nitrogen. The details of this process, however, form some of the most baffling problems in biochemistry. How, for instance, are the stable amino acids so readily decomposed *in vivo?*

What is the relation *in vivo* of ammonia to urea, and of both to the α-amino acids? The treatment of the subject falls naturally into four parts, the decomposition of proteins and amino acids a, by hydrolysis; b, by oxidation; c, by direct loss of ammonia; and d, by reactions not yet identified, but manifested by the detection of certain end products.

a. Hydrolysis. By the year 1886 the work of various investigators had established the fact that hydrolysis of proteins by acids led to the formation of ammonia, and it was generally agreed that the precursors of this ammonia were acid amides of unknown constitution, possibly acid amides of amino acids. Different proteins were found to contain characteristic amounts of this "amide" nitrogen. In 1886 Hirschler found that by the action of trypsin on fibrin ammonia was set free, and could be estimated. Hirschler was careful to exclude the action of micro-organisms. These results were confirmed two years later by Stadelman. It is perhaps a little curious that these authors did not follow up their results on trypsin by some experiments on the same lines with pepsin, but at all events nothing was published on this subject till 1899, when Zuntz showed that ammonia was set free by the action of pepsin on casein and serum albumen. One year later Jacoby made the discovery that ammonia was a product of the autolysis of liver. There was a very natural tendency to regard this not in the light of previous work on pepsin and trypsin as a hydrolysis of acid amides, but in the light of what was known of liver function, as a deamination of the amino acids set free in autolysis. Lang (1904) moreover found experimentally that liver tissue set free ammonia *in vitro* from added amino acids (though not so freely as from asparagine, the acid amide of aspartic acid). Bostock (1912) in part confirmed this, but recognized that the small amount of deamination of the amino acids which occurred "was not really representative of intra-vital changes." Asparagine she found to be deamidized freely, and this was all the more remarkable because *in vivo* the reverse was the case. A dog converted 96 per cent of the nitrogen in 10 grams of glycine to urea, and excreted it in 8 hours, but only 63 per cent of the nitrogen in asparagine under similar conditions, the remainder being excreted unchanged.

Between the years 1902 and 1911 Gonnerman (1902, 1903); Schwarzschild (1904); Lang (1904); Bergell and von Wulfing (1910); and Bergell and Brugsch (1910), all worked on amide hydrolysis by tissue enzymes *in vitro*. Some seventeen different amides and imides were tested, of which thirteen were found to be hydrolyzed by the liver, setting free ammonia, nine by the kidney, and three only by the pan-

creas. Under similar conditions the deamination of amino acids was negligible. These results revealed (as in so many other cases of enzyme catalysis) a marked specificity, and help to explain a series of papers by Henriques and Gjaldbak (1911, 1912) on the hydrolysis of proteins by pepsin and trypsin. These authors found more ammonia produced by pepsin than by trypsin during hydrolysis, but more amino nitrogen by the latter. They showed later that the ammonia produced with pepsin was due to the associated hydrochloric acid and they were evidently dealing with acid amides hydrolysed by acid but stable in the presence of pancreatic enzymes. Twelve years afterwards Luck (1924) isolated such an amide from caseinogen, and found that it consisted mainly of glutamin, and that it was decomposed by both liver and kidney but not of course by the pancreas. It may be mentioned that Hunter and Smith (1924) consider the amount of ammonia liberated by pure trypsin to be very small or even negligible.

This formation of ammonia by pepsin and trypsin helps to explain some of the recent work on blood ammonia, although Anderson and Roedmuller (1915) consider that the liberation of ammonia by these enzymes from proteins in the digestive tract is too slow to be of any importance. Bliss (1926) found more ammonia in the splenic vein, which helps to drain the stomach, than in the systemic veins, and pointed out that large amounts of ammonia are sometimes present in the stomach contents. Cholopoff (1926) found more ammonia in the portal than in the systemic veins, and noted that the ammonia came from both the small intestine and cecum. A discussion of the factors responsible for the ammonia values found in the blood was published in 1927–28 by the same author. Parnas and Klisiecki (1926) located the chief site for ammonia absorption in the cecum in rabbits, which may be put down to bacterial decomposition of undigested proteins in this organ, or to more rapid absorption of ammonia from this organ than from the higher parts of the alimentary canal. Which is the correct explanation could be determined by estimations of the relative amounts of ammonia in the various sections of the alimentary canal, and in the blood draining them. An additional reason for the high ammonia values found in the splenic and portal veins is the presence of urease in the stomach. This was discovered by Luck in 1924, and the action of gastric mucosa was found to resemble that of the vegetable urease in all respects. Any urea reaching the mucosa was at once converted to ammonia, and a solution of urea placed in the stomach caused an increase in the ammonia in the veins leaving the stomach (Luck, 1924b; Luck and Seth, 1925).

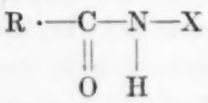
By 1913 it had become clear that the ammonia of autolysis was derived mainly from the same sources as the ammonia of peptic, tryptic, and acid hydrolysis—that is to say, from acid amides, and not from amino acids. Dakin had arrived at this conclusion from a study of kidney autolysis as early as 1904, but unfortunately used no other organs.

The catabolism of nuclear material provides another source of ammonia in autolysis. Nucleic acid contains amino derivatives of the purines, which may be attacked by enzymes either before or after their liberation from the nucleotides (Jones, 1920). Recently the suggestion has been made that ammonia formed in autolysis is almost entirely due to the deamination of these compounds (Gyorgy and Röthler, 1927). In evidence of this they found that in liver autolysis the ammonia produced naturally and the ammonia produced from added nuclear material were affected in the same way by pH and various inhibitory substances. Some of their figures are rather irregular, however, and their evidence seems to be insufficient to warrant a departure from the older view that the ammonia produced from nuclear material is only a small fraction of the total ammonia set free in autolysis. As far as the kidney is concerned, Dakin's work in 1904 seems to be conclusive evidence of the importance of the acid amides in this respect. Again, the optimum pH for ammonia production in their experiments on liver was 5–5.5. But McCance (1924) has shown that the optimum pH for ammonia production in autolysis is not the true optimum pH for any *one* reaction, but the pH at which at least *two* reactions, with optimum pH's to the acid and alkaline sides respectively can best combine. The digestion of boiled splenic or hepatic tissue by pancreatic enzymes leaves part of the ammonia precursor undecomposed. McCance took advantage of this fact to study the formation of ammonia by tissue enzymes, uncomplicated by the simultaneous hydrolytic cleavage, which is inevitable during autolysis. He showed thereby that whereas the optimum pH for autolysis was below pH 5 (Dernby, 1918), the optimum pH for ammonia production from the tryptic-stable filtrates of each organ was pH 7 (or higher). The same pH was found to be optimum for added amides. pH 5.5 (Gyorgy and Röthler) is therefore only an apparent optimum.

We know from the work of Osborne (1908–09), Luck (1924a) and others that the acid amides in proteins are mainly those of aspartic and glutamic acids (Coghill, 1926). Less work has been done on the decomposition of glutamin than asparagin because of the difficulty of procuring

the former, but we know something from Luck's work (1924b) of its decomposition by various glandular tissues. Enzymes capable of decomposing asparagin, and termed therefore asparaginases, have been found in the higher plants (Grover and Chibnall, 1927), in yeasts (Geddes and Hunter), fungi, and a number of animal tissues, including the human placenta (Maeda, 1924), but probably excluding the pancreas (Bergell and von Wulfing, Schwarzchild, Cathcart). Clementi (1922) has made a study of the distribution of this enzyme and found very much larger concentrations in the tissues of herbivorous animals than in those of carnivora and omnivora. He has suggested that its presence is verly largely a biological adaptation to foods. The suggestion seems scarcely warranted even by his own results, for the organs of carnivorous birds were found to contain large amounts of asparaginase. His experimental evidence moreover has not been confirmed on one or two tested points (Grover and Chibnall) and is in opposition to older work (von Furth and Friedman). From feeding experiments moreover asparagin is known to be decomposed by carnivora as well as by herbivora, and its nitrogen excreted as urea (Levene and Kober, 1908–09). Three of the four carbon atoms of asparagin are excreted as glucose by the phloridzinized dog (Nitsche).

Von Furth and Friedman, Dernby, and others pointed out that acid amides might be regarded as variations of the usual peptide linkage,



and therefore that the deamidization might be the work of the ereptases, which are known to be widespread in tissues (Grover and Chibnall, Waldschmidt-Leitz et al.). A considerable amount of work has been done under the assumption that the liberation of ammonia from acid amides is only a particular example of the general phenomenon of peptide hydrolysis (Levene, Sims and Pfaltz, 1926). Geddes and Hunter have completed a large amount of work on yeast asparaginase (including attempts to purify it) without apparently considering it as a protease. It is significant, however, that their purest preparation still contained active protease. Assuming the identity of deamidases with proteases, the apparent optimum pH for the ammonia production of autolysis is the result of the combined actions of the pepsin-like protease and the tissue ereptases (Hedin, 1923; Bradley, and Hertzman and Bradley, 1924–25). No work appears to have been done on the synthesis of

amides by tissue enzymes. This should yield fruitful results on the types of amide linkage in the various organs, and on their relative ease of formation.

It may seem a little inconsistent with the work just described on the inability of pancreatic enzymes to decompose glutamin and asparagin, to find that the pancreas autolyzing at pH 6 produces more NH_3 than the liver and much more than the kidney (McCance, unpublished work). It may be that the pancreatic amides being products of the synthetic activity of pancreatic enzymes are all available under changed conditions for their hydrolytic activity. The nature of these amides, however, and the cause of the apparent discrepancy are unknown.

By urine analysis it has always been shown that urea, not ammonia, is the chief end product of protein metabolism in mammals; and by feeding experiments that amino acids or ammonia introduced into the system are rapidly excreted as urea. Many workers have confirmed and extended the original experiments of Schutzen and Nencki (1872), and Salkowski (1875). Perfusion experiments, moreover, of Schröder (1882) and Salaskin (1898) indicated that the liver played a prominent part in the process, and this has been amply confirmed more recently by Löffler (1918).

The possibility of the formation of urea *in vitro* first attracted attention in 1894, when Richet, estimating urea by the hypobromite method, found that if a liver was washed and filtered, the amount of urea in the liquid filtrate increased on incubation in the presence of sodium fluoride. Later (1897) Richet and Chassevant claimed that this increase took place at the expense of uric acid, or added ammonium salts. Loewi in 1898 thought he found that, on incubating liver tissue, an increase of an alcohol-ether-soluble substance containing nitrogen occurred. His experimental results even led him to claim that an enzyme existed in liver which passed through a Chamberland filter, was not destroyed by alcohol, and which, with glycine and other amino acids as substrate, gave a large yield of this alcohol-ether-soluble material, estimated by the Morner-Sjoquist method. Ammonium carbonate was not attacked by the enzyme. The results of these two authors were contradictory to each other, and to much subsequent work, so that Löffler in 1918 was led to make the statement that no urea was produced in autolysis, or by tissue enzymes *in vitro*. The difficulty of estimating urea must be held responsible for some of the results on this subject which have not stood the test of time. Hammett in 1919 showed that pulped placental tissue gradually formed urea on incubation, and while suggesting the possi-

bility of this being due to arginase, he considered that it might be a synthesis from ammonium carbonate. In 1921, Fosse, exploiting his specific precipitant, xanthydrol, took this matter up once more, and published a paper with Rouchelman, showing quite clearly that urea was indeed a product of liver autolysis. There, however, they allowed the matter to rest, making no study of the conditions, especially with reference to the ammonia formed at the same time, and the relation between this ammonia and the urea. In 1925–26, Meyers, Ringer and Benson in a very brief report noted an increase of urea in autolyzing liver but not muscle tissue. Unfortunately neither Hammett nor these observers mention the pH at which they were working. Goldthorpe's publication (1925–26) is so fragmentary and his results so irregular that it is difficult to appraise their significance. Artom also has reported a formation of urea in kidney autolysis (1926), but his results are inconsistent. Absence of a proportionate consecutive increase or decrease in autolytic experiments is always highly suggestive of faulty technique.

In 1922 Marie reported that the addition of suitable amounts of adrenalin to an autolyzing liver doubled the amount of urea produced, and, assuming that the urea arose from carbohydrate oxidation in the presence of ammonia (Fosse, 1912), he suggested that the action of adrenalin was to increase the carbohydrate mobilization.

It is important to realise that while the formation of ammonia is a feature of any hydrolysis of proteins whatever the agent, be it pepsin, trypsin, hydrogen- or hydroxyl-ions, or the proteolytic enzymes of the tissues of animals, or plants (Castow, 1907), that of urea only occurs in the autolysis of certain organs. In addition to the increase in the liver reported by Fosse and Rouchelman (1921), and in the human placenta (Hammett, 1919), urea formation takes place in the mammalian kidney, spleen, and thyroid. No formation of urea has been detected in the pancreas, (ox, pig) lung, skeletal muscle (ox), or heart muscle (sheep): nor has it been found in the avian liver (hen) (McCance, 1924).

Knowing the important influence of pH on autolysis, and of an acidosis on the ratio of ammonia to urea excreted by the kidney, McCance (1924) began to investigate the factors influencing the production of urea in autolysis, and its relation to the ammonia simultaneously produced. Nash and Benedict had suggested that the ammonia excreted during acidosis was formed from urea in the kidney itself, and it was hoped to obtain some evidence on this point also.

Special studies were made of the liver, kidney and spleen. In these organs it was definitely determined that the formation of ammonia and

urea were independent phenomena for the following reasons: 1. Though in the spleen and kidney the optimum pH for both ammonia and urea production is pH 6, the optimum pH's for their production in the liver are not the same, that for urea being at pH 5.5, and ammonia pH 6. 2. Their rates of production varied quite independently. 3. The concentration of ammonia could be doubled by adding ammonium carbonate at any time during autolysis without affecting the smooth curve of urea formation. 4. The concentration of urea could be altered without affecting the formation of ammonia. There is therefore no ground for supposing that the urea is formed at the expense of the ammonia according to the laws of mass action, or that it is dependent upon it in any way. No urea was ever observed to disappear during the autolysis of the kidney or of any organ.

The fact that there is no mechanism for the formation of urea in the pancreas makes it possible to determine the true optimum pH for the production of urea in both the liver and spleen by following the action of their enzymes on the trypsin-digested organs at different pHs. Thus for the spleen:

	mgm.
A. Urea formed at pH 7 in 48 hours from substances in the pancreas-digested spleen, expressed in milligrams per 100 grams active autolysing tissue	94.5
B. The same at pH 6	8.4

and for the liver:

	mgm.
A. Urea formed at pH 6.8–7 from substances in the pancreas-digested liver, expressed in milligrams per 100 grams active autolysing tissue	376.0
B. The same at pH 5	117.0

A simultaneous autolysis of both spleen and liver at the same pH's showed, as always, a formation of more urea at the more acid pH. There must be a series of reactions before urea is set free, the observed optimum pH for urea production in autolysis being the reaction at which these several enzymes can act best.

The effect of adrenalin in promoting urea formation could not be confirmed with autolysing ox liver whether unbuffered or buffered to pH 5, 6, or 7, and adrenalin in very varying concentrations appeared not to influence the production of urea (Marie, 1922).

Since, however, it seemed that oxygen might increase the formation of urea by promoting the oxidation of amino acids, and possibly other

compounds (a subject which will be more fully considered later), the effect of oxygen and hydrogen gas on autolysis, and especially the formation of urea was next investigated.

The effect of these gases on autolysis had been studied by Laquer (1912), Bellazzi (1908), Morse (1916) and Shima (1922), the first of whom obtained marked inhibition of autolysis by oxygen, measured by total soluble and amino nitrogen. He used no buffers, and often oxygen under pressure. On the other hand the other three did not obtain this effect, and Morse (estimating total soluble nitrogen) certainly took up the work with the idea that oxygen might inhibit autolysis.

It was found that whereas hydrogen did not affect the production of urea in autolysis, oxygen, however prepared, inhibited it strongly. Nitrous oxide and other gases had no effect other than CO_2 which alters the pH. The conditions governing this inhibition and its nature were studied (1925), and it was found that the oxygen exerted a *partial* inhibitory effect on some stage in the process leading to urea formation. The effect on autolysis, however, was usually to obtain a smaller *total* amount of urea. This was due to other factors in the autolysing digest gradually inactivating the urea forming enzymes both in oxygen and hydrogen before the substrates were all decomposed. To obtain a smaller total amount of urea in autolysis in oxygen the process must be carried out at a pH at which the limiting factor in urea formation is the activity of these enzymes themselves.

The source of the urea remains to be discussed. The effect of oxygen, and the absence of relationship between the ammonia and the urea produced, exclude the formation of the latter from cyanate or carbonate, and Marie's theory that the urea has its origin in the oxidation of glucose seems untenable. There is, however, another well known source of urea.

It is generally agreed that the mammalian as distinct from the avian liver contains large amounts of arginase, which must account for some at least of the urea formed in such organs. Kossel and Dakin (1904) using isolation methods, Edlbacher (1915) and Fuchs (1921) using the Sorensen amino-N titration method for estimating arginine, all failed to detect arginase in the mammalian spleen. The same is true of Edlbacher and Röthler (1925).

The question therefore arises whether the arginine-arginase system can account for all the urea formed in autolysis. If this is so the following postulates should be fulfilled.

1. Only organs containing arginase should yield urea on autolysis. This so far as is known is correct. McCance was able to detect arginase in all the spleens he tested, but in a concentration so low that with the methods employed by other workers it might easily not be detected. Arginase has not been detected in the human placenta in which organ Hammett detected an increase of urea, but it is present in the placentae of lower animals (Edlbacher and Röthler). The striking example of the converse of this (organs without arginase being unable to form urea) is the avian liver (McCance, 1924).

2. No phenomena connected with urea formation in autolysis should be irreconcilable with what is known of the action of arginase. Thus whatever the *observed* optimum pH for urea production in autolysis, the *true* optimum pH is apparently not far removed from that of arginase which is pH 7 (Hunter and Morell, 1922). McCance was unable to demonstrate any inhibition of arginase by oxygen. He was, however, also able to show that the oxygen inhibition in autolysis did not concern arginase. For first, although it was easy to demonstrate an inhibition by oxygen of urea production from organs hydrolysed enzymically, it was not possible to obtain this effect from organs which had been completely hydrolysed by acids, although under these circumstances arginine was certainly present. Secondly, it was shown (unpublished experiments) that in those cases where the inhibition was obtained, arginase was not the limiting factor in the urea production. At the same time, some of the facts connected with this inhibition are difficult to explain if arginase is the sole source of urea.

3. No compound other than arginine should increase the urea production when added to an autolysing digest. This so far has been the case.

4. No more urea should under the best conditions ever be produced than can be accounted for by the amount of arginine present in the organ. This has not been tried, but Kossel's (1924) discovery of a specific precipitant for arginine will make it much easier to put the matter to the test.

Przylecki (1927) has considered the possibility of arginase being the sole source of urea in autolysis and neglected it, but his evidence is not sufficient to settle the point. He has also described a synthesis of urea from added ammonium salts under the influence of crude and semi-purified tissue enzymes. His experiments are not regular and show features not obtained by others. For example, out of 125 mgm. NH_3 added to a flask (weight of tissue not given) 75 mgm. disappeared, and urea synthesis only accounted for a fraction of the remainder. It is,

however, quite possible that an oxidative synthesis of urea may have been obtained (see later). Przylecki seems unaware of much of the work which has been published on this subject.

The rapid liberation of urea in the early stages of autolysis has been taken to indicate that arginine cannot be the precursor because in these early stages the liberation of amino acids in general has been quite small. (Mitchell and Hamilton, 1929.) This is not a valid argument for Hunter and Dauphinee have found that a very rapid cleavage of arginine from the protein molecule takes place under the action of trypsin. In the case of gelatin for example $\frac{1}{3}$ of the total arginine was set free in 30 minutes. There is no evidence at present of its rate of liberation by autolytic enzymes.

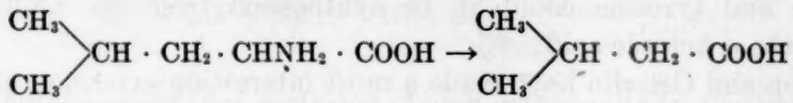
To recapitulate: Some of the nitrogen of proteins is liberated by the digestive juices and enzymes from the acid amides present in their molecules, although the amount of this is uncertain. Its occurrence is in keeping with what is known of the ammonia content of the blood in the portal veins. Ammonia is always formed during autolysis, partly from purines, but mainly from acid amides. During life the ammonia set free in this way is no doubt very small, but a study of its synthesis might assist towards an understanding of the structure of tissue proteins. Urea is also formed during autolysis in certain organs. Its origin is entirely hydrolytic. It remains to be proved whether arginine can account for it all.

b. Oxidation. There is an abundance of evidence from all sources that the metabolism of the α-amino acids may commence with the loss of the characteristic amino grouping by a process of oxidation. While this is a general statement, it has become abundantly clear since the pioneer work of Stolte (1903) that no two amino acids behave in exactly the same way, and the metabolism of each must be considered separately. Contrast, for example, the ability of iminazolyl lactic acid to replace histidine in a diet (Harrow and Sherwin), and the failure of ϵ-amino, α-hydroxy-caproic acid to replace lysine (McGinty, Lewis and Marvel). Under identical conditions *in vitro* amino acids show very different rates of oxidation (Robinson and McCance). The products of this oxidation, however, are not yet known with certainty, and different authorities hold very different opinions.

According to one view the result of this oxidation is the formation of ammonia and an α-ketonic acid, which then undergoes further decomposition by losing CO_2 with the formation of the next lower aldehyde. This on further oxidation gives the acid. This may for the moment be crudely represented thus:

$$R{\cdot}CHNH_2{\cdot}COOH + O \rightarrow R{\cdot}CO{\cdot}COOH + NH_3$$
$$\rightarrow R{\cdot}CHO + CO_2 + O$$
$$\rightarrow R{\cdot}CO{\cdot}OH$$

Evidence for this has been obtained in a variety of ways. Experiments on phloridzinised dogs showed that the α-amino acids behaved generally as though they were fatty acids containing one carbon atom less than the original amino acid. Embden on perfusing leucine through the excised liver actually isolated iso-valeric acid.

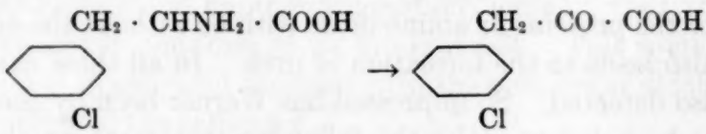


Leucine. Iso-valeric acid.

Friedmann and Maase showed that p. chlor-phenyl acetic acid was formed from p.chlor-phenyl pyruvic acid but not from p.chlor-phenyl lactic acid, throwing light on the intermediate stages. Otto Neubauer in 1909, assuming that homogentisic acid was a substance normally formed from tyrosine concluded that the keto-, and not the hydroxy-acid, was the intermediate step; because feeding the former but not the latter to an alkaptonuric patient led to an increased excretion of homogentisic acid. The same observer isolated l.mandelic acid from a dog's urine to which he had fed d.-phenyl-amino acetic acid. This change could best have been brought about by the formation of the keto- from the α-amino acid and the asymmetric reduction of the latter. Thus:

$$C_6H_5CHNH_2{\cdot}COOH \rightarrow C_6H_5CO{\cdot}COOH \rightarrow C_6H_5{\cdot}CHOH{\cdot}COOH$$

Flattow in 1910 fed meta-tyrosine and m.chlor-phenyl alanine to dogs, and isolated from the urine the keto-acid in each case.



Neubauer and Fromhertz (1911) satisfied themselves that the keto acid was the compound directly formed from the amino acid in the production of fusel oils by yeasts, and that any α-hydroxy acids isolated were formed by secondary reduction of the ketonic acid. The existence of an enzyme capable of splitting off CO_2 from ketonic acids is also some indication that these acids are normal products of metabolism. Finally, the most convincing evidence of all comes from the synthetic

experiments of Knoop (1910, 1911) and Embden and Schmitz (1910, 1912). The former showed that on feeding γ-phenyl α-keto-butyric acid as the ammonium salt, the α-amino acid (as the acetyl derivative) was excreted, while from γ-phenyl, α-hydroxy-butryic acid only traces of the α-amino acid were isolated.

$$C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH \rightarrow C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH$$

Embden and Schmitz by liver perfusion showed that alanine, phenylalanine and tyrosine could all be synthesised from the ammonium salt of the α-ketonic acid.

Knoop and Osterlin have made a most interesting extension of these experiments by making a study of this synthesis *in vitro*. It is brought about by the action of reducing agents in the presence of ammonia and the keto acid. The most interesting of the reducing agents used was cystin. There can be no doubt in the face of all this evidence that the amino acids can be oxidised with loss of ammonia under natural conditions, or that the change is a fairly general one (Dakin, 1922).

On the other hand Werner has accumulated a mass of chemical evidence that the formula usually assigned to urea is wrong, and that free urea possesses a ring structure. Werner has shown that the synthesis of this urea consists of a combination of ammonia and cyanic acid, and that all previous syntheses purporting to establish the carbamide structure were really manifestations of this cyanic acid synthesis. While confirming and extending early work of Bechamp (1856), Ritter (1871), and Hofmeister (1896), Werner and Fosse (1912, 1919 and 1921) have shown that the oxidation of glucose, fructose, formaldehyde, acetaldehyde, glycerol, succinic acid, proteins and amino acids, etc., by alkaline permanganate in the presence of ammonia gives considerable yields of urea (Werner, 1923). These authors have also shown that oxidation of the proteins or amino acids with or without the addition of ammonia also leads to the formation of urea. In all these cases cyanic acid was also detected. So impressed has Werner been by these results that he has been led to make the following statement on the subject of the formation of urea *in vivo*. "Cyanic acid must be formed in the liver, otherwise urea would not be found there." (Werner, *Chemistry of Urea*, Chap. xii.) Further, he adds: "In the various amino acids derived from the proteins we have the groups $-H_2C-NH_2$, $=HC-NH_2$, $\equiv C-NH_2$, and $=HC-NH-$, which could readily yield $O=C=NH$ (cyanic acid) as the final stage of their oxidation." Then "Cyanic acid is quantitatively hydrolysed to urea by water alone." "Why then should Nature proceed to the formation of such end products

as CO_2 and NH_3 with the . . . . production of ammonium carbonate, to be followed by a process of dehydration before urea is ultimately formed?" Werner explains how perfusion of ammonium salts can lead to a large formation of urea by a comparison of the two equations

(1) $2HN = CO + HOH \rightarrow HN : C(O)(NH_3) + CO_2$
(cyanic acid)

(2) $HN = CO + NH_3 \rightarrow HN : C(O)(NH_3)$

It should be noted that perfusion of ammonium salts on this view can never do more than *double* the previous rate of urea production. Werner's views may then be summarised thus: 1, he denies the possibility of obtaining a synthesis of urea *in vivo* other than through cyanic acid; 2, he suggests the path of synthesis of this cyanic acid from proteins; 3, while not absolutely denying that ammonia is formed from the amino acids *in vivo*, and offering an explanation of the conversion of any ammonia into urea in terms of cyanic acid, he affirms that the latter is the normal product of the biological oxidation of the proteins. To deal first with his suggested method of synthesis of the cyanic acid: this implies that the α-carbon atom of the amino acid is split off with the $-NH_2$ group attached to it whereas the evidence is overwhelming that this is not the case. Much of the evidence has just been quoted and it is sufficient here to repeat that all the biological evidence indicates that α-amino acids undergo α-oxidation with ultimate formation of the next lower fatty acid, which must contain the carbon atom to which the $-NH_2$ group was originally attached. The evidence therefore indicates that Werner's suggestion is wrong. Fearon and Montgomery have made another suggestion, namely, that the amino acids are oxidised in the form of an internal salt. This would explain the formation of cyanic acid together with the next lower aldehyde, but not the formation of the keto-acid.

$$R \cdot \underset{\underset{CO—O}{|}}{\overset{|}{CH—NH_3}} + O \rightarrow R \cdot CHO + HNCO + H_2O$$

Their results, however, did not lend much support to the theory and there is no biological evidence of its occurrence.

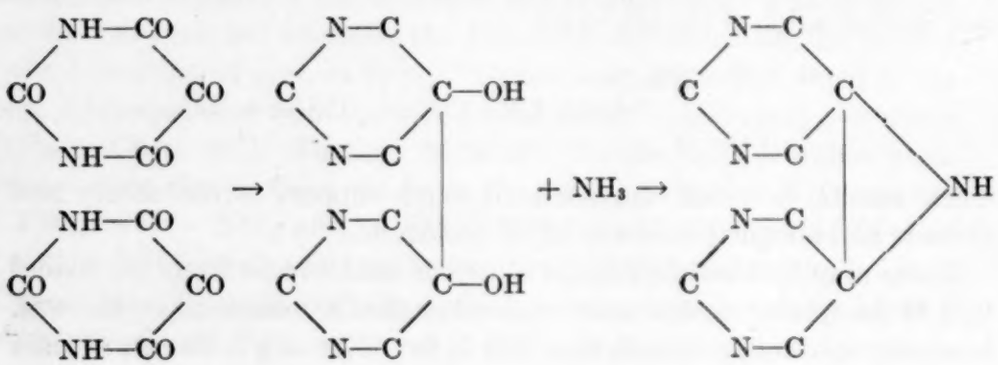
There may be some formation of cyanic acid *in vivo* from the oxidation of acetylated amino acids and other similar compounds; there is, however, no direct evidence that this is so. Nothing is known of their

oxidation, but such acetylated compounds may certainly be formed in very diverse forms of life (Neubauer and Warburg, 1911; Fromhertz, 1911; Knoop, 1922) and *in vitro*, Fearon and Montgomery (1924) have shown that the oxidation of such acids gives a much higher yield of cyanic acid than oxidation of the simple amino acids. A critical survey of the evidence, however, shows that in spite of Werner's assertions to the contrary there seems to be no good reason to abandon the older theory that the liberation of ammonia with formation of the keto acid is the path of oxidation of the amino acids *in vivo*. Further evidence that the older view is the correct one comes from a study of the known mechanisms of oxidation of α-amino acids available *in vivo*. Dakin pointed out that hydrogen peroxide + iron salts could oxidise amino acids with the formation of the next lower aldehyde, + CO_2 and NH_3. *In vivo* the aldehyde would undergo oxidation to the next lower fatty acid. The enzyme peroxidase occurs widespread, and now that Thurlow (1925) has discovered a source of hydrogen peroxide in the cell, Dakin's work assumes a new importance (Harrison and Thurlow, 1926; Harrison, 1927). The oxidising agent which Fosse and Werner have used almost exclusively is alkaline permanganate which is quite unphysiological. There are other methods of oxidising α-amino acids which give the same results as the hydrogen peroxide method. These must now be discussed as they throw light on the chemistry of the rough equation

$$R{\cdot}CHNH_2{\cdot}COOH + O \rightarrow R{\cdot}CHO + NH_3 + CO_2$$

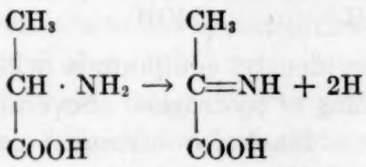
and the general nature of peroxide oxidations.

Strecker (1862) originally showed that the α-amino acids treated with alloxan yielded the next lower aldehyde, carbon dioxide, and ammonia, and Traube confirmed this and replaced alloxan by quinone (1911). Both these reactions take place in the absence of oxygen, and Strecker states that murexide is formed. This takes place as follows:

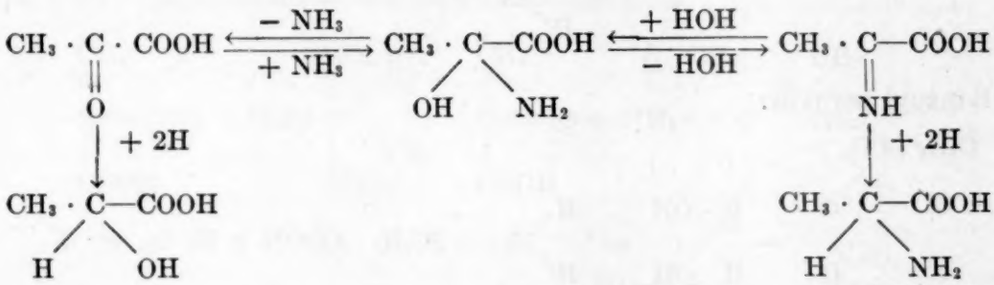


and shows that the first action of alloxan must be the removal of two hydrogen atoms from the amino acid.

Warburg showed that amino acids underwent oxidation on biological lines on the surface of blood charcoal (1921) (also Toyoda, 1927). Warburg always laid the greatest stress on the activation of oxygen by the iron present (1924), but Wieland (1924) has recently shown that the same results could be obtained in a vacuum with charcoal in the presence of di-nitro-benzene (which of course could not be supposed to be activated by iron) or if palladium black replaced the charcoal. This makes it certain that the removal of two hydrogen atoms from the amino acid must be the first stage. Thus:



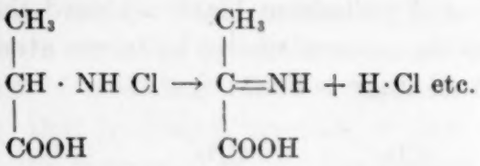
Knoop and Osterlin (1925; 1927) have recently provided definite evidence of the importance of the α-imino acid in the synthesis, and therefore presumably the breakdown of the amino acids. They showed that if methylamine replaced ammonia synthesis of N-methyl α-amino acids followed, but no synthesis took place if dimethyl- or trimethyl-amine were used. In these cases the hydroxy acid is the chief yield. Thus starting from the keto-acid:



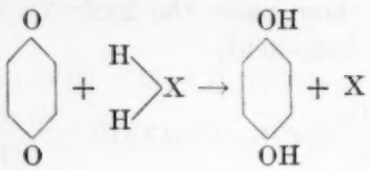
The equations show that for the synthesis to proceed two of the groups attached to the nitrogen atom must be labile, and this explains why no synthesis takes place with di- and tri-methylamine. The ease with which the keto and amino acids are interconverted according to the conditions is explained by the small changes of energy involved. Wieland (1924) has shown that 1 gram mol. of alanine in passing to pyruvic acid loses only 16.4 Cals., i.e., only 5.7 per cent of the heat of combustion

of pyruvic acid. Similar arguments to the above have led Knoop to stress the importance of the imino acids also in the synthesis of acetylated compounds.

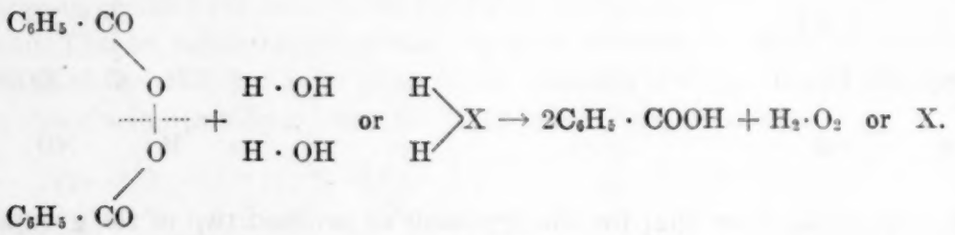
It is of interest here to recall some of the work of Langheld in 1909. This observer showed that chlor-amino acids on warming decomposed into the next lower aldehyde, carbon dioxide, ammonia and hydrochloric acid. Now in this case loss of HCl is the initial step. There is no oxidation to take place.



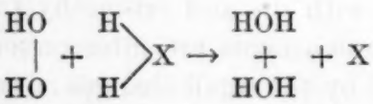
α-amino acids are evidently compounds which under the correct conditions lose two atoms of hydrogen. Several non-specific catalysts (charcoal and palladium black for instance) can render these same hydrogen atoms more labile. A peroxide oxidation shows itself to be the removal of two hydrogen atoms from the oxidized compound, and it is instructive to regard peroxides in this light, namely, as hydrogen "collectors," combining the properties of an enzyme which can "activate" hydrogen, and molecular oxygen or methylene blue which can "accept" hydrogen so activated. Thus quinone:



Benzoyl peroxide:



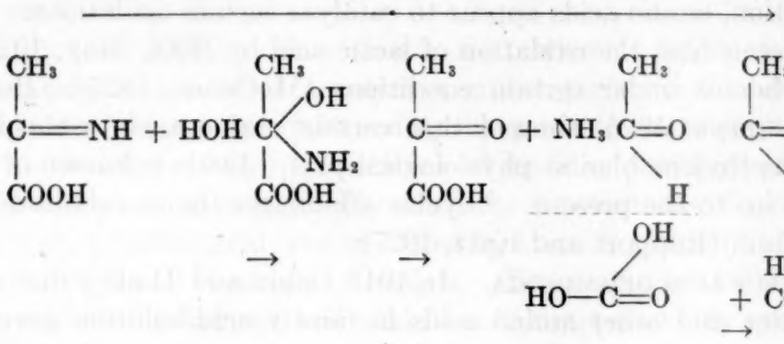
Hydrogen peroxide:



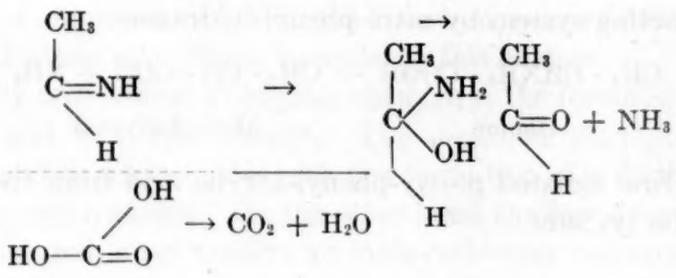
Oparin has investigated an auto-oxidizable compound occurring naturally in the vegetable kingdom which catalyzes the oxidation of

amino acids (1921, 1927). This compound, chlorogenic acid, is a catechol derivative (Gortner, 1908a, b), and the catechol is probably the active group. The autoxidation is very slow at pH 7 but is catalyzed by tissue enzymes. It proceeds rapidly by itself at pH 9. Reduction is relatively independent of pH. The yields of ammonia are quantitative and it is certain cyanic acid plays no part in this oxidation as it would be fixed as cyanate at pH 9, and if hydrolyzed yield urea and not ammonia. The oxidations of amino acids secondary to the oxidation of certain phenols (Chodat and Schweitzer, 1913; Happold and Raper, 1925, Pugh and Raper, 1927), are also to be regarded as peroxide oxidations, and ammonia the form in which the nitrogen is separated. No cyanate could be detected by McCance (1925) among the products, but the yield of free ammonia is not quantitative probably due to side reactions. Neuberg and Kobel (1927) have described an oxidation of amino acids by methyl glyoxal which they regard as analogous to the Strecker reaction. The yields of free ammonia were small, and their explanation that the ammonia set free reacts with excess of glyoxal not altogether satisfactory, for the yields of ammonia were good when purines were being oxidized by the same reagent. This reaction requires further investigation.

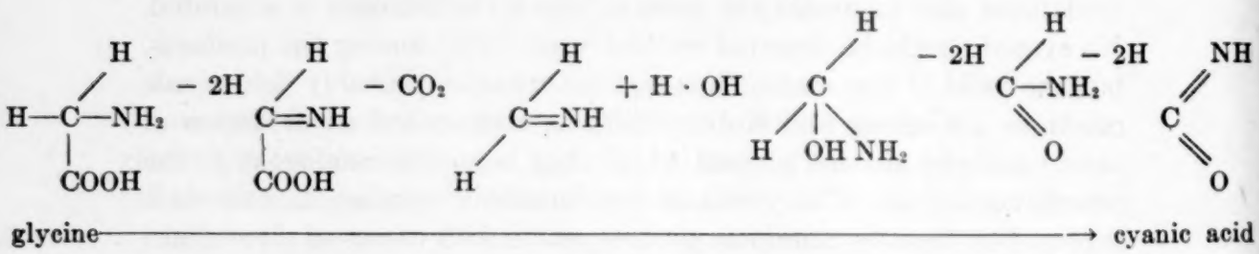
The α-imino acid, formed by the loss of two hydrogen atoms from the corresponding α-amino acid, probably decomposes in the following way. Wieland (1924) states that this part of the reaction is also catalyzed by charcoal but proceeds *in vitro* by itself.



or



The essential features of the above equations are the loss of CO_2 (carboxylase) by hydrolysis, and the fact that C=O is more stable under the existing conditions than C=NH. Under other conditions decarboxylation of the imino acid may occur and would lead by reduction to the formation of a primary amine. This may be the mechanism of the formation of these compounds which are of considerable biological importance. It is clear that there is no room in this scheme of things for the formation of cyanic acid in Werner's sense, but it is easy to see how glycine could give rise to it, as Fearon and Montgomery found (1924).



This may also explain why the keto acid is not the normal path of breakdown of glycine (Dakin, 1922), but the path of formation of glucose from glycine is still obscure.

Little is known of the path of breakdown of the nitrogen atoms in the ring of histidine and tryptophane. It seems certain that they are ultimately excreted as urea (Konishi, 1925).

In certain circumstances possibly connected with their specific dynamic action, amino acids appear to catalyze certain oxidations. Thus glycine accelerates the oxidation of lactic acid by H_2O_2 (Ray, 1923–24) and of phenols under certain conditions (McCance, 1925). Borsook and Wasteneys (1925) showed that certain amino acids with glucose reduced methylene blue at physiological pHs. Little is known of these reactions up to the present. Glycine stimulates the metabolism of an isolated limb (Rapport and Katz, 1927).

C. Direct loss of ammonia. In 1913 Dakin and Dudley discovered that alanine and other amino acids in faintly acid solution gave considerable yields of methyl-glyoxal, if the latter was continually removed from the reacting system by nitro-phenyl-hydrazine:

$$CH_3 \cdot CHNH_2 \cdot COOH \rightarrow CH_3 \cdot CO \cdot CHO + NH_3$$

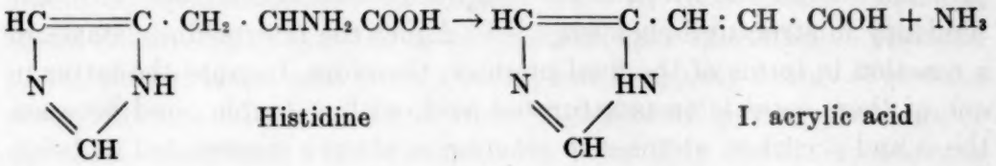
Alanine Methyl glyoxal

In 1921 Hirai isolated p.-oxy-phenyl-acrylic acid from the action of B. Proteus on tyrosine:

$$HO \cdot C_6H_4 \cdot CH_2\,CHNH_2 \cdot COOH \rightarrow HO \cdot C_6H_4 \cdot CH : CH \cdot COOH + NH_3$$

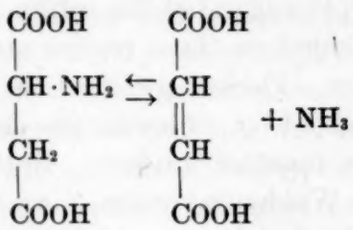
Tyrosine — p. oxy-phenyl-acrylic acid

and Raistrick found that histidine gave rise to iminazolyl-acrylic acid by bacterial action:



Histidine — I. acrylic acid

Quastel and Woolf have found that fumaric and aspartic acids are intimately related in the presence of intact "resting" microörganisms, and have definitely proved the existence of the following equilibrium:

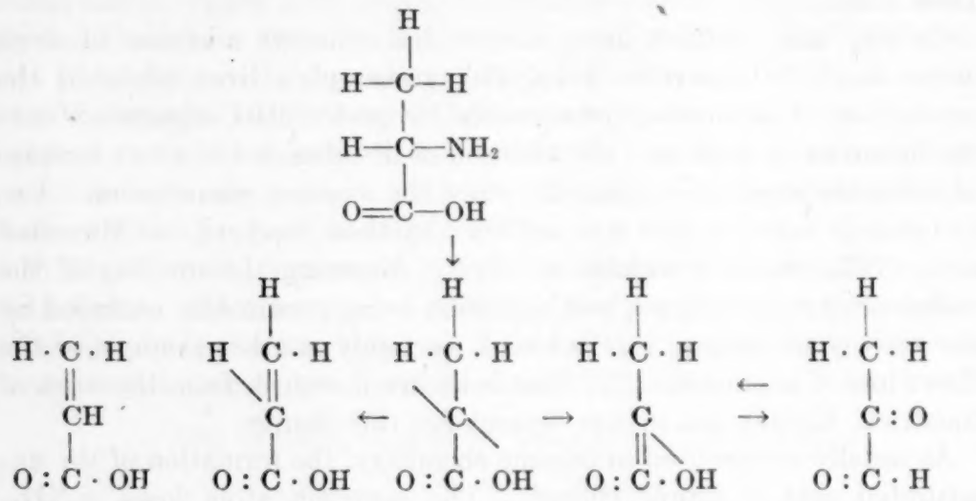


Kotake and Konishi have shown that iminazolyl-acrylic acid is excreted by dogs after feeding l-histidine. The direct removal of ammonia from the α-amino acid is the one step which is common to each of these cases.

György and Röthler have shown that whereas addition of most amino acids to anaerobic autolyzing guinea-pig's liver inhibited the production of ammonia, (presumably by preferential adsorption onto the deamidizing protease) the addition of histidine led to great increase of ammonia production especially when the reaction was alkaline. Unfortunately aspartic acid was not tried by these workers, but Meyerhof et al. (1925) found it to have no effect. Knowing the stability of the amino acids to hydrolysis, and oxidation being presumably excluded by the absence of oxygen, this is almost certainly another example of the direct loss of ammonia. Histidine is known moreover from the work of Raistrick, Kotake and others, to undergo this change.

As usually represented in organic chemistry, the formation of the unsaturated acid is simple enough. The α-carbon atom loses $-NH_2$, and the β-carbon atom $-$ H, with the formation of a double bond between the α and β atoms. On the other hand the formation of glyoxal, however pictured, must involve an intra-molecular rearrangement, and

there is no experimental evidence whatever as to the structure of the compound first formed from, and actually in equilibrium with, the amino acid. An important link is missing here. Considering the structural formula of alanine, we may assume with certainty that in the direct loss of ammonia the radicle $-NH_2$ leaves the molecule as a whole. The problem is to decide which of the hydrogen atoms accompanies it. The tendency in structural chemistry is to depict the intermediate stages of a reaction in terms of the final product; therefore, because the latter in one of these cases is an unsaturated acid, with a double bond between the α and β carbon atoms, the reaction is always represented as being the loss of an $-H$ from the β atom and $-NH_2$ from the α atom. It is as reasonable to suppose that both $-NH_2$ and $-H$ are split off from the α carbon atom. This supposition is of interest in connection with the Walden inversion (Fischer, 1907). To explain this Fischer (1911) and Werner (1911) suggested the intermediate formation of an unstable addition compound on the α carbon atom, not bound by the ordinary laws of valency. Fischer criticised the theory of the Walden inversion passing through a C =, or methylene stage on the ground that this could only give an inactive product. Both the $-NH_2$ and H– groups, however, in the Walden inversion, may leave the same carbon atom not simultaneously, but consecutively, with intermediate intramolecular rearrangement. (See Nef, 1908.) This assumption at once explains how both the glyoxals and the unsaturated acids can be formed from α-amino acids by loss of ammonia. Thus:



Which of these actions takes place in excess probably depends very largely on the conditions, but in one form or other this decomposition of the α-amino acids is a widespread one, occurring in the most diverse spheres of life. In 1911, for example, Erlich and Jacobsen isolated large amounts of d.p.-oxy-phenyl-lactic acid from a culture of the mould Oidium lactis growing on l.tyrosin. In 1925 Kotake, Chikano amd Ichihara found that the same organism gave quantitative yields of p.-oxy-phenyl-lactic acid from tyrosin, but that only traces of p.-oxy-phenyl-lactic acid were formed from p.-oxyphenyl-pyruvic acid under the same conditions, which excludes the possibility of the formation of the d-hydroxy acid from l-tyrosine by oxidation of the α-amino group and asymmetric reduction of the p.-oxy-pyruvic acid. Hence this must be a case of the direct loss of ammonia from tyrosin with formation of the glyoxal and subsequently the α-hydroxy acid (not the hydrolysis of tyrosine, as Kotake, Chikano and Ichihara supposed, because Erlich and Jacobsen showed that the *d*.p-oxy-phenyl lactic acid was obtained from the *l*-tyrosin). This series of reactions commencing with the direct loss of ammonia from an amino acid followed by the formation of the α-hydroxy acid from the glyoxal probably represents the course of events in all those cases where the α-hydroxy, rather than the α-ketonic acids have been shown to be stages in the catabolism of a particular amino acid e.g. phenyl alanine. (Mori (1922), Embden and Baldes (1913), Kotake and Okagawa (1922), Kotake, Masai and Mori (1922).)

As long ago as 1879, Baumann showed that a mixed culture of bacteria formed p-oxy-phenyl-propionic acid from tyrosin, and many other similar reactions have since been discovered. Thus alanine gives rise to propionic acid (Brasch, 1909), valine to isovaleric acid (Neuberg and Karczag, 1909) and histidine to iminazolyl propionic acid (Ackermann, 1910). Many other examples of this reaction are given by Dakin, 1922, and others have been reported since this date (Speakman, 1926; Quastel and Woolf, 1926). All these reactions may be brought about by the direct loss of ammonia and the reduction of the unsaturated acid. This is at any rate a simple way of explaining such a change. It has been proved to occur in the case of the formation of succinic from aspartic acid (Quastel and Woolf; Cook and Woolf) and all the steps have been separately shown to occur in the case of histidine.

The other three possibilities are:

1. Direct reduction with the addition of two hydrogen atoms and separation of ammonia:

$$R \cdot CH \cdot NH_2 \cdot COOH + 2\,H \rightarrow R \cdot CH_2 \cdot COOH + NH_3$$

This is possible and seems to have been generally assumed in the past. It is very difficult to effect *in vitro* (Dakin, 1922).

2. Hydrolysis to the hydroxy acid and reduction of the latter. The formation of the hydroxy acid in this way in unlikely (Dakin, 1922).

3. Oxidation to the ketonic acid followed by reduction through the hydroxy acid. This is a usual method of formation of the hydroxy acid (Dakin, 1922) but with the hydroxy acid the reduction generally ceases.

These possibilities could be tested out by trying the action of the various tissues and organisms concerned on the synthetic intermediate compounds concerned in each case, as has been done with so much success in the case of the hydroxy acids (Neubauer and Gross, Neubauer and Fischer, et al., Dakin, 1922).

It is interesting to note in this connection that iminazolyl acrylic acid will to some extent prevent the loss in weight of rats due to the removal of histidine from the diet (Harrow and Sherwin). Iminazolyl lactic acid prevents any loss of weight and restores growth again. Iminazolyl pyruvic acts less readily than I. lactic in this respect. If this is interpreted to mean that I. lactic is the normal intermediate metabolite of histidine, then the probable path of metabolism is 1, direct loss of ammonia with formation of the glyoxal; 2, formation of the lactic acid from this. If oxidation to the keto acid were the first step followed by reduction, then I. pyruvic should replace histidine even better than I. lactic. The use made of I. acrylic acid may suggest some normal decomposition along these lines.

Sufficient evidence has been adduced to show that this reaction (the direct loss of ammonia from an α-amino acid) may be a general one, although the evidence at present is derived mainly from a study of histidine, aspartic acid, phenyl alanine and tyrosin. It is impossible to say what percentage of the ammonia set free *in vivo* is formed in this way. Quastel and Woolf have shown that in one case at least synthesis of an amino acid may take place by this method under conditions of enzymic catalysis, and the possibilities thus opened up for exploration are great. Possibly, for instance, synthesis of this sort might be obtained by perfusion. However this may be, it remains for the present the least known aspect of amino acid metabolism.

d. DECOMPOSITIONS OF UNKNOWN NATURE. Of considerable theoretical interest is the formation of ammonia on muscular contraction or trauma described by Parnas and Mozolowski (Parnas, Mozolowski and Lewinski, 1927). According to Parnas (1928) this production of

ammonia is irreversible (Nachmannsohn, 1928) and proportional to the tension and lactic acid generated at the same time. The absence of oxygen does not inhibit the production of this traumatic ammonia. Embden and his co-workers (1928) disagree with these views. They have shown that the ammonia set free during contraction undergoes resynthesis during recovery and that it bears no relation to the lactic acid. Embden believes that the adenine-pentose-nucleotide isolated by himself from muscle is the sole precursor of this ammonia. Enzymes effecting this deamination have been separated from muscle tissue and studied (Schmidt, 1928). The product of their action is inosinic acid, the corresponding hypoxanthine-pentose-nucleotide, known for nearly a century (Liebig) and of well established constitution (Haiser; Neuberg and Brahn; Levene and Jacobs). There is every reason to believe that this system accounts for some of the ammonia set free from muscular sources, but according to Parnas it can only represent about $\frac{1}{3}$ of the ammonia liberated on trauma. Embden emphasizes the fact that in frogs seasonal influences may greatly modify the results. The Parnas school have shown that enough ammonia is set free during severe muscular exertion in man to raise the ammonia in the blood draining the part from 2 to 8 times (Parnas, Mozolowski, and Lewinski). No variations in urea accompany the ammonia increases, which have also been shown to have no connection with cyanate. The precursor, apart from the adenine nucleotide, is unknown and also the mechanism of its decomposition.

The ammonia produced by surviving muscle (Meyerhof, Lohmann and Meier, 1925) would appear to be due to an oxidation, probably of amino acids, and the same may be true of the ammonia set free from nerve tissue both at rest and during activity (Gerard and Meyerhof, 1927; Winterstein and Hirschburg, 1926). Rosch and Kamp (1928) have shown that ammonia is set free on illuminating the retina but that although the retina can deaminize adenine nucleotide with liberation of ammonia, this is not the source of the ammonia set free on illumination. The mechanism is probably one of oxidation. According to Lee and Tashiro surviving muscle produces much less ammonia than does nerve, when compared weight for weight. The processes are physiological for they depend upon the integrity of the tissues. These observers do not consider that adenine is the precursor. They have calculated that the ammonia produced by the human organism in this way may amount to between one and two grams daily, figures of considerable quantitative significance. The nature of the production

of ammonia by various tissues reported by Warburg, Posener and Negelein (1924) remains for the present obscure. It depends apparently to a limited extent upon the presence of oxygen and to be connected with the power of the tissue to produce glycolysis.

The ammonia in the blood. Henriques and Gottlieb (1924) and Fontes and Yovanovitch (1925) consider that there is no ammonia in the peripheral blood. Were this correct it would be impossible to reconcile it with the work of others on the various decompositions of protein previously mentioned. Other workers, however, do report a definite amount of ammonia in peripheral blood. (Parnas and Klisiecki, 1926a, b; Parnas and Heller, 1924; Russell, 1923; Gigon, 1926; Klisiecki, 1926; Luck and Seth, 1925; Benedict and Nash, 1926; Bliss, 1926; Parnas, 1927, and others.)

During life the liver is able to remove practically all the ammonia from the circulating blood as it passes through provided its oxygen supply is adequate, and this explains the low concentration normally found in the peripheral circulation. The small amount of ammonia in blood makes it certain that the kidney must manufacture the large amounts of ammonia which it may excrete from some precursor (Rabinowitch, 1924, 1926; Loeb et al., 1924 Benedict and Nash, 1926; see also Hendrix and Bodanski). Nash and Benedict (1921) originally suggested that urea might fulfill this rôle and this view has been supported by the careful experiments of Mann and Bollman on hepatectomised dogs in which injection of urea led to increased excretion of ammonia. If this is correct it is curious and interesting that no urease has been demonstrated in the kidney. Benard and Besancon have concluded from perfusion experiments which are not described in sufficient detail that the kidney cannot convert urea to ammonia. Other possible precursors are amino acids and acid amides (Bliss, 1929) or even some compound not yet described.

In the blood of some animals, notably the rabbit and man, but to a much less extent in the dog, a non-colloidal substance occurs limited mainly to the corpuscles which distintegrates on standing under the influence of a corpuscular enzyme setting free ammonia. The optimum pH for this decomposition is pH 8, and it is independent of the presence of oxygen. Two milligrams of ammonia per 100 cc. of human blood may be set free in this way. The precursor is not urea nor cyanic acid as has been suggested by Fearon (1926); Mozolowski and Taubenhaus (1927). The ammonia is not set free from amino groups. From healthy blood the amount of ammonia set free in this way is very constant, but

it is lowered in cases of hepatic deficiency and in cases where large amounts of ammonia are being excreted continuously in the urine. There is therefore evidence that this precursor may give rise to the urinary ammonia, in spite of the fact that dogs whose blood contains so little, may excrete large amounts of ammonia. Of its nature nothing is at present known, but further information is eagerly awaited. (Luck and Seth, 1925; Adlersberg and Taubenhaus, 1926; Parnas and Taubenhaus, 1925; Taubenhaus and Sternberg, 1927; Klisiecki, Mozolowski and Taubenhaus, 1927; Adler and Schwerin, 1927.) Its properties recall those of ergothioneine, the betaine of thiolhistidine which has been isolated from blood (Newton, Benedict and Dakin, 1927; Eagles and Johnson, 1927). A point rather against ergothioneine being the precursor of the blood ammonia is the fact that the concentration of the former varies greatly, while that of the latter is very constant during health. There is some evidence that some at least of the ammonia precursor may be found in the muscles (Adlersberg and Taubenhaus, 1927).

CONCLUSION

The formation of urea. Whatever the intermediate stages this is the fate of nine-tenths of the nitrogen of protein metabolised by mammals.

The experiments of Bollman, Mann and Magath are so definite that we must assume that the liver is the only site of urea formation (at any rate in dogs) and that it is also the chief site of deamination. This anatomical association is in keeping with the low concentrations of ammonia in the blood. It appears, however, that embryonic kidney tissue growing *in vitro* has the power of forming urea in certain circumstances (Holmes and Watchorn, 1927a). There is evidence that in birds the two processes, deamination and urea formation, are to a greater extent anatomically dissociated, only the latter being confined to the liver. (Falkenhausen and Siwon, 1925; Minkowski, 1886.) If Werner (1923) is correct in thinking urea can only be formed through cyanic acid (and his chemical evidence is very strong) the best view of its formation is that the liver can oxidise ammonium salts through a cyanate stage. In support of this is the failure of the liver to withdraw ammonia from the blood if even partially asphyxiated, and the known occurrence of this oxidation under approximately physiological conditions. (Abderhalden and Buadze, 1926; Fichter and Kern, 1925.) The absence of comparatively gross amounts of cyanate in the blood is

no objection (Gottlieb, 1926). Montgomery, 1925; Fearon, 1926; (compare the minute concentration of ammonia in the blood.) But the reaction of the animal to cyanate injections, the failure of cyanate to relieve ammonia poisoning, and its toxicity (Gottlieb, 1926) are very difficult observations to reconcile with this theory. It would seem, however, that cyanic acid may be the precursor of the urea (and ammonia) formed by embryonic kidney tissue growing *in vitro*, though the general significance of the part played by the hydantoins in giving rise to the cyanic acid is obscure (Holmes and Watchorn 1929). It is certain that urease has no synthetic action in animals (Luck and Seth, 1925). That it is an integral part of the mucosa of the stomach, however, and not introduced with the food is shown by the fact that it only occurs in localised areas of the stomach wall. Fearon has attempted to explain the action of urease in terms of cyanate but these views have been severely and justifiably criticised and the difficulty is admitted by Fearon himself. (Kay, 1923; Sumner, 1926; Mack and Villars, 1923; Artom, 1926, 1927; Fearon, 1926a, b.) The balance of evidence is certainly in favour of the carbamate path of breakdown.

Interesting experiments have been reported by Iwanoff and his coworkers on the formation of urea in mushrooms. This takes place at the expense of amino nitrogen but only in the presence of oxygen, and it is depressed by the presence of carbohydrates, which are presumably preferentially oxidised (cf. Holmes and Watchorn, 1927b). The concentration of ammonia in these plants is never high but it is stated that the synthesis of urea takes place through the ammonium carbonate stage. These experiments open up a most promising field of research on the biological formation of urea. While the reactions offer close analogies to those of the higher animals they take place in a closed system comparable to the developing egg but capable of more rigid experimental control. With improved technique much of interest may be forthcoming from this field.

It may well be that in nature the path of synthesis of urea in the liver and elsewhere is not the same as the path of breakdown by urease, but at present the observations are so contradictory that no one theory can embrace them all. The last word has certainly not been said.

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CONTRACTURES OF SKELETAL MUSCLE

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When a muscle is stimulated through its nerve or by induction shocks directly applied it passes into a state of activity revealed by an electrical potential change, the development of heat and tension, and the accumulation of catabolic products. A single momentary stimulus causes a wave of contraction to pass along the muscle, and the muscle as a whole gives a twitch. Repeated stimuli cause a series of twitches, and if these be made to come sufficiently close together a sustained contraction results. There is no longer any sign of discontinuous activity in the mechanical response; but, that discontinuity exists, is clearly manifest in the waves of electrical change coursing throughout the length of the fibers. To be contrasted with such contractions, which we may consider as due to *complete* involvement of the muscle, are certain states of sustained shortening or tension development in which the list of signs is *incomplete*. These are generally characterized by a mechanical change which is not propagated nor attended by a wave-like electrical variation; but, in the condition produced by acids there are probably still greater deficiencies. The one type of response is called a tetanus, the other a contracture.

While the word contracture as originally used had a precise though limited meaning, it has come to be a term without definition, convenient in its vagueness for the designation of various states of muscle shortening, themselves not well understood but in which there is a real or fancied difference from tetanic contraction. It has been applied to conditions as far apart in fact as decerebrate rigidity and the fibrous degeneration following too tight bandages, with a resulting confusion which suggests the advisability of abandoning the word altogether. The term is, however, too thoroughly incorporated in the physiological literature in a more or less precise sense to make abandonment practicable, and one must therefore choose the other horn of the dilemma, definition.

A review of the literature discloses the fact that a large number of related muscular states may be included under the term "contracture"

provided the following restrictions are imposed for qualification under such a designation:

1. At least some part of the normal contractile mechanism of the muscle must be involved. As will be seen later, in most contractures the muscle mechanism is very extensively involved. Tension, heat and lactic acid are produced, and the active portion of the muscle becomes negative. Only conduction of the mechanical response and a wave-like action potential are missing. It would therefore make greatly for simplicity if the term contracture could be limited to mean these states alone; but such a restriction would exclude the condition produced by acids (and possibly other substances) as they, according to widely held current opinion, act directly on some specifically contractile structure in the muscle, without the intervention of the usual metabolism. However, such an exclusion would be inadvisable on account of long usage. On the other hand, because of the different nature of the muscle shortenings produced by acids it would be well, for the sake of clearness, always to refer to them as acid-contractures.

Permanent shortenings in which the muscle is completely at rest would be excluded; so also would shortenings which could be produced with equal facility in connective tissue, as for instance by temperatures of 60° to 70°.

2. The change in the muscle must be reversible. Contractures as produced by the majority of agents are initially fully reversible, but they later become complicated by irreversible changes so that relaxation does not occur when the agent is removed. The contracture is said to pass into rigor. This fact necessitates a statement of the relation between the two. If rigor be due to a continuation of the process which produces the contracture, it is of importance in the elucidation of the latter; but if it be an independent phenomenon, it is outside the scope of this review. The relationship was originally clarified in 1890 by Bernstein, who wrote an interpretation of some experiments performed by two of his pupils, Klingenbiel and Morgan. The latter found that the contracture produced by strong ammonia soon relaxed again; the muscle became non-irritable but was still transparent. In contrast with this a chloroform contracture remained fixed at its maximum, but the muscle became cloudy and the proteins coagulated. If, now, chloroform were added to the ammonia muscle, protein coagulation occurred but no shortening.

Bernstein, therefore, concluded that the contracture was not the result of coagulation but was attributable to a contraction process which

he considered to be identical with that occurring normally. In rigor the muscle was shortened only because coagulation had "surprised" it in a state of contracture and fixed it so that it could not relax with the passing of irritability. In 1919, after considering the evidence which had accumulated in the intervening twenty-seven years, v. Fürth again expressed the same opinion as to the relationship between contracture and rigor. Accordingly, in this review only the initial reversible changes will be treated as pertaining to contracture.

Reversibility alone, however, cannot be considered to be a perfect criterion of a contracture as it is quite possible that changes leading to shortening in structures separate from the proper contractile mechanism may be initially reversible.

3. Tetani are excluded. While this limitation easily excludes many contractions, it is often hard to apply, both on account of the technical difficulties involved in determining the existence of the necessary conditions for a tetanus, and because of the fact that a tetanus and a contracture may co-exist.

As an alternative to the word "contracture" the expression "tonic contraction" is often used, largely because of the slowness of the muscle response; but the latter term is even more unfortunate than the former because of the unjustifiable implied connection with tonus. No contracture has been shown to function in the movements of the body or in the maintenance of posture; nor has one been demonstrated to be under control of any of the coördinating mechanisms of the animal organism. Moreover, even if such an instance should definitely be found, the employment of the expression for all forms of contractures would not be justified.

Modes of production. The forms of energy which are commonly known to act as tissue stimuli are also capable of inducing contractures. Thus the latter can be brought about by electrical, thermal, mechanical and chemical means.

The general plan of treating the subject in this review will be first to outline the characteristics of the contractures produced by these various agents. Then the individual aspects of muscular activity, as they are present in the several forms of contracture, will be analysed with the end in view of building up a compositive picture of a contracture.

Contractures produced by electrical stimulation. In contractures from all causes the magnitude of the contracture curve as compared with a control twitch or tetanus is greatly dependent upon the type of lever used. When the contracture is compared with a twitch it is of course

essential that the twitch be recorded with a lever having a period sufficiently short to prevent over-shooting. Otherwise the twitch height appears to be relatively too great in comparison with the contracture height. Unfortunately in many cases such a precaution has not been taken. Comparisons made with the tetanus height are generally better, as the fling error is thereby eliminated; but there is still another factor of great importance which must be considered in interpreting such observations: namely, whether a light balanced isotonic lever or a tension lever has been used. A record inscribed by a light isotonic lever tells a different story than one inscribed by a tension lever. This is because relaxation of a muscle is passive. As was shown in the old experiment of Kühne (1859, p. 418) a muscle caused to contract on the surface of mercury does not again relax. Without the pull exerted by a lever or at least by the weight of the more dependent portions of the muscle itself, restoration to the original length is impossible, in spite of a complete return of the muscle to its resting state. Thus, when working against a very light lever, a muscle which has once contracted may remain shortened, although it is exerting a very small force. Furthermore a muscle may achieve a considerable degree of shortening when it could develop but little tension; so, as Bethe (1923) says, the shortening does not give a dependable picture of the forces which the muscle could exert. These are much better expressed when the muscle is contracting against a tension lever. Then the degree of shortening is also much less dependent on the previous activity of the muscle, and more dependent on the forces exerted at the moment. Therefore small contraction remainders do not appear as shortenings of considerable height. Relatively few data have been obtained with tension levers but those which have been are very instructive.

The first contractures were observed in fatigue curves. These were described in detail over half a century ago and they are annually recorded by the thousand in class laboratories. Nevertheless many of the features which they manifest are still obscure.

When a very irritable muscle is stimulated with successive supramaximal break induction shocks so as to give a series of twitches, it often happens that during the first contractions the relaxation is incomplete. The level of the bases of the separate twitches progressively rises, but a maximum is soon reached and succeeded by a fall. Then a second period ensues during which the base line again rises; and this rise may now persist throughout the experiment, though its magnitude and the degree to which it is maintained depends upon the lever. It is

large and well sustained when opposed only by a light lever, smaller and very rapidly receding with a tension lever. Finally, when the stimulation is stopped, the lever, particularly if light, returns to the base line only after some time. All of these phases, the initial rise, the secondary rise and the final failure to return at once to the base line are called contractures in the literature. Actually they are different things. The first rise is always absent on indirect stimulation and is easily fatigued; the second rise occurs after both indirect and direct stimulation and is a product of fatigue; the final failure of relaxation also occurs after both forms of excitation but may be much larger after a direct tetanus.

Depending upon the irritability of the muscle and the conditions of the experiment either of the two rises of the base line just described may appear without the other; and as they do not mean the same thing they must be differentiated. But this has not always been done in published experiments, with the result that in the absence of the necessary data differentiation is practically impossible. The two rises show too much superficial similarity.

The fact that an unfatigued muscle may remain somewhat contracted between twitches was first noted by Kronecker, but the condition is generally known as Tiegel's contracture, the terminology following a later description in which for the first time the designation contracture was used as opposed to contraction. According to Tiegel's description the contracture has the following characteristics. It may be produced in excised muscles or in muscles under their normal circulation. It occurs best in early spring frogs. It is produced only by direct stimulation and its magnitude depends upon the strength of the stimulus. Appearing first notably when shocks maximal for the muscle as a whole are applied, it increases with the strength of stimulus until it may reach a height greater than the original twitch. In a series of twitches at two second intervals it disappears after about 100 twitches, due to fatigue.

Thus Tiegel has clearly described the first rise of the base line in a "fatigue" curve.

Tiegel's contracture has also been shown very beautifully in man by Mosso. Mosso's fatigue curves of the flexor muscles acting on the middle finger, as recorded with his ergograph, are often cited; but the curves which he obtained on direct stimulation and which differed very greatly from the ones made during voluntary innervation, are much less known. When the muscles were stimulated directly contracture developed during the first six or seven contractions and at times reached a height much greater than that of the first contraction of the series.

On further stimulation, however, the contracture fell off although the contractions continued to increase in size. The magnitude of the contracture augmented with the strength of stimulus. On cessation of stimulation at the height of contracture, relaxation occurred very slowly and during this period the force exerted by the muscle was often by no means inconsiderable, for a weight of 2.5 kgm. could be held by a muscle shortened 6 mm. There was of course, the possibility that, as the experiment was performed by Mosso, reflex innervation occurred; but such an objection has been cleared away by some recent experiments of Schäffer (1920). The latter observed that the contracture as described by Mosso still occurred after the brachial plexus had been blocked by novocaine. He also found that the phenomenon was very dependent on the condition of the muscle, being greatly reduced when the latter was rendered anemic.

The electrical stimulus most favorable for the production of a contracture was found by M. Lapicque and Weill to be one which is short with respect to the duration of a twitch contraction, and thus with respect to chronaxie. It would therefore necessarily be very strong. The contracture produced was not identified with that of Tiegel; but the conditions of the experiment indicate that such might be the case.

The form of the twitches produced by strong break induction shocks was found by Schenck (1895) and by F. B. Hofmann and Flössner to be veratrin-like, and the latter also observed that the contracture is a local phenomenon confined to the region of the physiological cathode. They compared it with the effect produced by a constant current which, according to Wundt, is a sustained contraction lasting throughout the period of current flow. This too is strictly localized. It occupies a region of microscopic dimensions in the region of the cathode, when the current is minimal, but grows to considerable proportions as the latter is increased (Wundt).

The absence of conduction is best seen in Biedermann's experiments on a frog sartorius fixed at its middle point and stimulated at the ends (1895, p. 159). On the cathode side there was a twitch followed by a sustained contraction, on the anode side a twitch alone. This was in a fresh muscle. When the muscle was thoroughly fatigued the stimulus produced a sustained contraction without the twitch, and since this was in the cathode end and not conducted, the opposite end of the muscle remained quiescent. A similar contraction unaccompanied by a twitch was also produced by Saito in anesthetized muscle; and recently Bremer (1928, c) has succeeded in showing in irritable muscles of *rana temporaria*

that constant currents of near threshold strength may produce a contracture—the sustained contractions of the older observers were certainly contractures—without the usual accompanying twitch. The chronaxies of the contractures were about 100 times that for a twitch of the same muscle.

Both Biedermann and Saito observed that the contracture lasted only during the passage of the current and noted that the possibility of attributing the shortening of the muscle to injury and swelling was precluded by the rapidity of reversal.

It will be seen in a later section that Tiegel's contracture, which is responsible for the *first rise of the base line* in a fatigue curve, is due to a prolongation of energy mobilization by the muscle. The *second rise* is connected with the relaxation-phase. The latter is prolonged, as has been known since Helmholz, and this prolongation permits an incomplete tetanus to occur at a rate of stimulation which produces discrete twitches in a fresh muscle. The relaxation is also incomplete as may be demonstrated by stopping the stimulation. If the muscle be lightly loaded a small residual shortening persists which Hermann (1879) called a "contraction remainder." Of these two alterations the slowing of relaxation is the more important. What is produced is essentially a summation, and therefore to refer to the second rise of the base line as a contracture is to name it after the more insignificant factor contributing to its existence.

In extreme fatigue the contraction remainder grows until it is capable of exerting a tension equivalent to 15 per cent of the normal maximal tetanus tension; and since it is apparently due to acid accumulation it would be a contracture of the acid type. However, all the contractures persisting after a tetanus or a series of twitches cannot be considered entirely as contraction remainders, since those following supra-maximal direct stimuli may be attended by a continued increase in the lactic acid content of the muscle (Meyerhof and Lohmann, 1925). Their mechanism is similar to that obtaining in Tiegel's contracture and they can be so designated.

Contractures from indirect stimulation. Following nerve stimulation acid-contractures or contraction-remainders are of course possible. But Tiegel's contracture could not be produced indirectly by Tiegel himself, nor ordinarily by Beritoff. Beritoff, however, observed that if winter frogs were warmed to 22° a single shock applied to the nerve produced a veratrin-like twitch in the muscle. The veratrin-like portion of the response fatigued after a few repetitions but recovered

when the muscle was allowed to rest. Bremer (1928, a) found that *rana temporaria* and the toad differed in some respects from *rana esculenta*. In all three a single indirect stimulus produced only a twitch, but in temporaria and the toad two stimuli at an interval of 2–5σ caused the twitch to be followed by a contracture, which from its appearance and fatiguability seemed identical with Tiegel's contracture. He explained the necessity for a double stimulation in the light of an observation made by L. Lapicque and the Chauchards; namely, that a tissue, having a chronaxie much larger than the nerve innervating it, can only be excited through its nerve by a summation of stimuli. Bremer's experiments suggest that indirect post-tetanic contractures may not always be contraction-remainders (1928, d). An analogy between the contracture produced by indirect stimulation ("neuromuscular contracture") and the behavior of the muscles in Thomsen's disease is drawn by the author (1929).

The Vulpian-Heidenhain-Sherrington phenomenon is another indirectly induced contracture but it differs entirely from the preceding instances. It is neither mediated through the motor nerve nor preceded by a twitch. In 1863 Philippeaux and Vulpian made the remarkable observation that after section and degeneration of the hypoglossal nerve, stimulation of the lingual nerve, which normally has no motor effect on the tongue whatever, caused a contracture. Vulpian later traced the action to the chorda tympani. No effect was produced when the lingual was stimulated above the point of entrance of the chorda nor after the chorda fibers had been caused to degenerate.

Heidenhain examined the contracture in more detail. About four days after the hypoglossal nerve had been sectioned the muscle fibers were seen to be in active fibrillation; then the lingual nerve, which up to this time had been without action, began to assume its new properties. Signs of contracture on stimulation of the lingual developed between the fourth and the sixth day, increased to the eighth or ninth day, remained constant until the end of the third week and then began to decrease, coming to zero in sixty-eight to ninety-eight days, when the hypoglossal had regenerated. Weak stimuli merely increased the fibrillation, but stronger ones produced a general contracture. With the head occiput down, stimulation caused the tongue to curl upward, rise to the floor of the mouth and deviate toward the side of the stimulation. In other words, it took the same position that it would have taken on stimulation of the hypoglossus.

The contracture itself had properties strikingly different from the

contraction produced through the proper motor innervation. The tongue responded only when much stronger shocks were applied to the lingual nerve than would be necessary for the hypoglossal and then after a prolonged latent period. While the latter for the hypoglossal was 0.02 second, for the lingual it was at least 0.08 second. Ordinarily it was several tenths of a second and with weak stimuli whole seconds. The contraction time was longer, amounting to 0.45 to 1.33 second as compared with 0.045 to 0.06 second on direct stimulation. The contraction outlasted the stimulus and the relaxation was very gradual. The force of the contraction was weaker. Single induction shocks produced only a slight localized fibrillary contraction, but if repeated at intervals as long as one second they gradually led to a sustained contraction. Repetition at shorter intervals made the rate of onset more rapid.

The chorda stimulation produced a marked vasodilatation in the tongue, as shown by its "flaming red" color. The movement, however, was undoubtedly not due to the dilatation. In the first place it could be seen through the mucous membrane that the tongue muscle fibers, were contracting; in the second place contracture could be produced in the excised tongue; and finally vasodilatation occurred without contracture, when the tongue muscle was in the relaxation phase following a nicotine injection.

The next one of these "pseudomotor" innervations was discovered by Rogowicz in 1885. He severed the facial nerve at the stylomastoid foramen and found that 12 to 14 days later stimulation of the subclavian loops caused a contraction of the upper lips and, at times, of the orbicularis palpebrarum. This was confirmed by Van Rijnberk.

Finally in 1894 Sherrington rediscovered the phenomenon in the muscles of the leg and thus established it as a general proposition. He cut the 6th and 7th lumbar and the first and second sacral spinal roots between the ganglia and the cord, and then two or three weeks later stimulated the peroneal nerve with very strong faradic shocks, producing thereby a slow dorsal flexion of the ankle and extension of the toes. The contracture outlasted the stimulus for five seconds, then the muscle slowly returned to its resting length. Sherrington felt that the muscle could not have contracted as the result of spread of stimulus as it occurred when there were 200 mm. of nerve conduction and disappeared when the nerve was crushed.

The facts as they have been historically outlined have been confirmed many times (Van Rijnberk, Frank, Nothmann and Hirsch-Kaufmann,

Dale and Gasser, Hinsey and Gasser). Further studies have also been made in search of an explanation of the occurrence. There are two problems in connection with the nervous pathways involved: one, as to the fibers that must degenerate before the muscle is sensitized; the other, as to the fibers mediating the contracture on stimulation of the nerve.

The sympathetic nervous system. The independence of the "pseudomotor" phenomenon from the sympathetic nervous system has been established by Van Rijnberk's experiments. The tongue phenomenon cannot be brought about by ablation of the cervical sympathetic nor is stimulation of the sympathetic after degeneration of the hypoglossus of any avail. The same relationship is also true for the limb muscles. It is a matter of indifference for the sensitization whether or not the sympathetic is intact, and contraction does not occur after root section when the abdominal sympathetic is stimulated. Only in the case of the Rogowicz experiment is the sympathetic involved.

Sensitization of the muscles seems to result from degeneration of the motors fibers. In the hypoglossal nerve there are motor fibers and, according to Boeke and Langworthy, proprioceptive fibers; the facial nerve has a similar composition; and in the Sherrington experiment the motor fibers and the central branches of all the sensory fibers are cut. Van Rijnberk, after finding that sensitization did not take place following degenerative section of the motor roots alone, pointed out that this would necessarily mean that there are fibers traversing the dorsal roots from the spinal cord and that these fibers must be severed. The necessity for such a radical assumption, however, was eliminated when Hinsey and Gasser repeated the experiment with a positive result.

Stimulation. When the chorda tympani is stimulated both afferent and efferent fibers are involved, and there is thus an element of uncertainty as to the fibers responsible for the tongue phenomenon, which is not present in the case of the leg phenomenon where the contracture can only be mediated through axons of dorsal root origin. In both instances very strong induction shocks are necessary for stimulation; and Bremer and Rylant have shown that the fibers excited in the chorda have a long chronaxie. Both of these facts indicate that the fibers are small and that such is the case has been shown by Hinsey and Gasser. They analysed the electromyogram of the sciatic nerve of the cat with the aid of a Braun tube oscillograph, during the induction of the Sherrington phenomenon, and found that the contracture did not appear until after the action potential, as it was then known, had reached a

maximum. They therefore attributed the activity to fibers smaller than 5μ as the action potential in the nerve stimulated by currents subthreshold for the Sherrington phenomenon was large enough, according to the analysis of Gasser and Erlanger, to indicate the involvement of all the fibers larger than this. The large proprioceptive fibers are thus definitely eliminated as would be expected from their absence in the chorda. That they might cause contraction of the intra-fusal fibers is also impossible for another reason. The latter could not produce the tensions encountered (116).

Further analysis demands a knowledge of the histology of the nerve endings in muscle; in fact one could hardly find a better example of a problem demanding for its solution the coöperation of histologist and physiologist. However, only a few points in the histology can be mentioned in this review and for a more comprehensive treatment of the important and difficult experiments in this field the reader is referred to the critical resumés of Boeke (1927) and Hinsey.

Boeke has described in the tongue fine accessory endings which remain in the position of the motor terminations after degeneration of the hypoglossus. These he attributed to the chorda tympani as they seemed to degenerate with the latter, and he thought they were derived from the sympathetic nervous system. Under similar conditions Langworthy found, in the cat, fibers associated with the blood vessels only; but in the rat there were branches from the blood vessel plexus to the musculature ending there in dustlike endings. He attributed these to the cranial autonomic system (1924).

The opinions as the origin of the fibers have been expressed without proof, so it is actually impossible to say whether they are motor or sensory, sympathetic or bulbar autonomic. Furthermore Hinsey points out the possibility that the accessory fibers ending on muscle may be as yet undegenerated collaterals of somatic fibers. He also calls attention to the fact that many of the fibers appearing to be on muscle may be on the capillaries. The chance of such a confusion has also been pointed out by Langley (1921, 71).

Boeke associates the Vulpian phenomenon with his accessory endings and this is the explanation which is favored by Van Rijnberk and Langworthy. Aside from any anatomical objections which might be raised against this view there are several physiological difficulties. If the endings be sympathetic as Boeke supposes them to be, then, in view of the apparently conclusive nature of Van Rijnberk's observations as to the negative effect of sympathetic stimulation, they cannot be

the ones concerned. If they are concerned they cannot be sympathetic, although they might be autonomic, as held by Langworthy. Also for the accessory endings to be involved at all it would be necessary to assume an entirely different mode of action from the one known for the normal ending, because of the form of the mechanical response to stimulation and because the rhythmic electromyogram to be expected in a tetanus is, according to Schäffer and Licht, absent.

In the leg phenomenon the situation is clearer as the fibers to be sought must be small dorsal root fibers. The dorsal root contains a large number of small fibers both myelinated and unmyelinated (Ranson, 1921). Unmyelinated fibers of dorsal root origin have been described in muscle by Kulchitsky and others. They cannot be numerous, however, as according to Langley (1922) but few unmyelinated fibers of any kind go to the gastrocnemius of the cat. Fine myelinated and unmyelinated fibers of dorsal root origin were seen by Hinsey to end in the perimysial connective tissue near blood vessels and in the adventitia of blood vessels, but not hypolemmally in muscle fibers. This being the case the contracture must surely be, as Heidenhain said, pseudomotor.

In the pseudomotor theories, as opposed to those based on an accessory innervation, speculation has centered largely around the vasodilatation which accompanies the contracture. Vasodilatation, per se, was eliminated by Heidenhain because the condition could be produced in the surviving tongue; but this fact did not eliminate complicity of the vasodilator fibers. Rogowicz brought the lip phenomenon into connection with vasodilatation by pointing out that in the dog, on which his experiments were performed, the vasodilators run in the subclavian loops (Dastre and Morat); and he made the generalization that all muscles after excision of their motor nerves respond with contracture to stimulation of their vasodilator nerves. This view met no contradiction after the discovery of the leg phenomenon as the existence of vasodilator fibers in the dorsal roots has been without question since the experiments of Stricker and of Bayliss (1901).

Heidenhain supported the association of the tongue phenomenon and vasodilatation because they both have a very similar course. When the lingual nerve is stimulated strong shocks are required and the responses appear only after a long latent period and outlast the stimulation. Recently Hinsey and Gasser (1930) have adduced additional evidence of another nature in the case of the spinal dorsal roots. Erlanger and Gasser had shown that, when the action potential of the dorsal root is recorded with high amplification, a wave appears in the

potential picture entirely separate from the main sensory wave even when the distance of conduction is short. This wave, which they labelled C, is made up of fibers having a different set of constants from the main wave and has a velocity of conduction of about two to three meters per second. With this in mind, Hinsey and Gasser measured the leg volume of the cat when the roots were stimulated and compared the vascular reaction with the electrical response of the root; and they found that as the strength of stimulation was increased the appearance of dilatation occurred very precisely at the time that the C wave appeared in the action potential. They then repeated the experiment on the Sherrington phenomenon and found that here too the appearance of contracture was exactly coincident with that of the C wave. These facts taken together strongly support the notion that the contracture is dependent on the vasodilator mechanism.

A number of suggestions have been made as to what the interrelation may be. One is that the vasodilator nerves in their course through the muscle stimulate the muscle fibers with their action currents, but the impossibility of this is proven by the ineffectiveness of the larger action currents from the spindle fibers and by the existence of a smooth electromyogram. Some other connection must therefore be sought and the only one which suggests itself is a chemical one. Langley (1921, p. 77) expressed the opinion that the pseudomotor effect is probably due to an action of metabolites on the altered muscle, but unfortunately it is hard to tell what he had in mind in making the statement. More recently Bremer and Rylant, Hinsey and Gasser, and Dale have in turn independently arrived at the conclusion that the correlation must be humoral. A substance is supposed to be liberated in the terminal mechanism of the vasodilator nerves which can affect the muscle when sensitized by denervation. It is a well known fact that denervation of smooth muscle renders it more irritable to drug stimulation (Elliott, Anderson); and the removal of the motor innervation of striated muscle must have some analogous effect to account for its sensitization. Skeletal muscle is known to develop susceptibilities to drugs, which it does not possess normally, and it might become sensitive to a chemical substance formed in the blood vessels.

The question remains as to what the nature of the substance might be. Lewis and Marvin have presented good experimental evidence that antidromic dilatation of the capillaries is hormonal and they suppose the substance to be histamine-like. Histamine, however, could play no part in the Sherrington phenomenon as it is entirely without a contrac-

ture-producing action in denervated muscle (49). A substance having all the necessary pharmacological properties would be one which is nicotine-like, as nicotine is a vasodilator which is not antagonized by atropine (Ranson and Wightman)—a necessary condition as antidromic dilatation is not so antagonized (R. Hunt)—, and it stimulates denervated muscle. But, no such substance is known to exist in the body. On the other hand Dale and Dudley in an important communication have demonstrated that acetylcholine is a normal constituent of the body and Dale now proposes that acetylcholine may be the substance sought. In every detail but one this proposal is beautifully in accord with the facts. Acetylcholine liberated from the region of the blood vessels, perhaps the arterioles, would act on the muscle cells to produce contracture, like acetylcholine added from without; and it would not be antagonized by atropine. The difficulty arises in connection with acetylcholine as the humoral substance responsible for dilatation. When it is added from without its vasodilatory action is vigorously antagonized by atropine whereas antidromic dilatation is not (R. Hunt). To surmount this difficulty Dale has proposed that the acetylcholine as it is physiologically formed may be in a position not accessible to acetylcholine.

Heidenhain remarked after his study of the Vulpian phenomenon, "Paradoxien kommen in dem Organismus nicht vor; wo sie scheinbar auftreten verbergen sich hinter ihnen noch unbekannte Principien allgemeiner Natur." The succeeding development of the subject now indicates that the unknown element lies in the physiology of the peripheral blood vessels. The muscle fibers merely act as an indicator for the hormone hypothetically formed. There is at present no evidence that muscle can nornally be made to contract by stimulating the dorsal roots, and it is normally unaffected by acetylcholine. This fact affords a serious objection to the assumption of a secondary tonomotor innervation of muscle through the dorsal roots as has been done by E. Frank and others.

CONTRACTURES PRODUCED BY THERMAL STIMULATION. When a muscle is subjected to too high a temperature many irreversible changes take place, and the muscle goes into rigor, but these changes do not bear directly upon the problem of reversible contractures. The latter can, however, be produced in muscle by heat as was shown by Gotschlich. The most extensive observations have been made by Jensen. By plunging a sartorius muscle through hot Ringer's solution into cold he was able with a minimum of irreversible injury to induce a contracture

with a tension as much as half of that of a tetanus. These contractures were not conducted and could be produced in muscles rendered non-irritable to electrical stimulation by narcosis or by previous freezing and thawing.

CONTRACTURES PRODUCED BY MECHANICAL STIMULATION. The "ideomuscular" contraction of Schiff (1859) produced by a blow or stroke cross-wise of the muscle fibers has often been likened to the contractures electrically excited (e.g., Hermann, 1876). It can be evoked in a narcotized muscle (Saito).

CHEMICAL CONTRACTURES. Kühne (1860) who was the first systematically to study pharmacodynamic contractures, made the remark that the whole host of chemical substances stands at the disposal of the physiologist for this purpose. Such is indeed the case; compounds of the most varied chemical properties, when applied to skeletal muscle, produce responses essentially the same in kind. There are detailed differences in the pharmacology, to be sure; but these differences are largely outside the scope of this review. What is of more importance is those similarities in action which shed light on the nature of the contracture process itself: similarities which are in fact sufficiently great to render classification on a physiological basis difficult. The main group of contracture-producing substances, if applied in sufficient concentration, is directly effective on skeletal muscle, wherever found. But there are two exceptions to the rule. The action of some substances is manifest only after a twitch, and the actions of others is dependent upon the existence in the muscle or parts of the muscle of a special receptive mechanism. Accordingly, for convenience in discussion, the chemical agents have been divided somewhat arbitrarily into three groups.

I. SUBSTANCES PRODUCING CONTRACTURE IN ALL MUSCLES. *Acids.* The contractures produced by acids have been studied more intensively than those of other substances on account of their bearing on the effect of the physiologically produced lactic acid.

From the researches of D. Dale and Mines, Burridge, Kopyloff and particularly Schwenker the following characteristics are known. If a muscle be thrust into an acid bath there is an initial rapid shortening which comes to a maximum and is followed, often after a slight relaxation, by a second and slower shortening. The same initial maximum also occurs in tension curves (vide Bethe, 1923, fig. 3). According to Schwenker, if the muscle be washed during the first rapid rise the reversal is prompt and complete; if washed at the initial maximum but

before the second rise, there is again a prompt recovery, but it is incomplete and the residuum is very slow to disappear. If the second slow rise be permitted then only the slow form of recovery is possible. As the contracture progresses electrical irritability disappears, and when this stage is reached reversal is no longer possible although the shortening or tension production progresses. But even at the contracture maximum which is irreversible the muscle has not developed all the tension of which it is capable, for Bethe (1923) finds that the latter can be still further increased by chloroform.

No definite evidence has been produced as to the nature of the sequence of events. Burridge suggested a surface and a deep effect, but the two parts seem to differ in kind as well as in time of appearance. The form of the contracture curve suggests a summation of two processes: the one a short process rapidly reaching a maximum and then declining, the other a process slowly approaching a maximum which is sustained; and the idea suggests itself that the first one is akin to a physiological contraction, while the other is the expression of secondary changes of varying degree of reversibility leading to a permanent setting of the fibers.

Acid contractures are not conducted when one end only of the muscle is poisoned (Wilmers) and they occur in narcotized muscle (Bethe, Fraenkel and Wilmers).

That a relation exists between the strength of the acid and the amount of the contracture was first shown by D. Dale and Mines. At a given molar concentration the stronger acids cause a greater contraction than the weak ones; however when solutions of the same reaction are compared the weak acids are the more effective. HCl is quite without effect at a pH which, if produced by a weak acid, would cause a strong contracture (Schwenker). The situation is quite in keeping with the action of acids elsewhere, for instance the toxicity to infusoria (Barratt, Collett) and the effect on the taste buds (Richards, Kahlenberg). It may depend on two factors. One was suggested by D. Dale and Mines, namely, that when the hydrogen ions are supplied by a weak acid the pH rises more slowly as the ions are combined with the tissue than when they are supplied by a dilute concentration of a strong acid. The other factor is the greater permeability of the cell for weak acids. Strong acids do not in general penetrate cells until there is injury of the plasma membrane and irreversible changes are under way. For instance, impermeability to strong acids or alkalies has been shown for paramoecia by Harvey, for sea urchin eggs by Warburg and for medusa

by Bethe (1909). Muscle contracture is not produced by HCl weaker than 0.001 N and at that concentration the injury must be considerable as Grant observed that the response to electrical stimulation falls off very rapidly at reactions more acid than pH 4. In fact, this is the reaction at which HCl has recently been observed by Komiyama to enter heart muscle; and at this point irreversible contracture begins.

Alkalies like acids cause a contracture in two phases. The contracture of the second phase is not always maintained, however, particularly after strong concentrations of ammonia. The muscle relaxes and is then no longer excitable by electrical stimuli, by chloroform or by heat (Klingenbiel, Rossi 1910). In other words, a physical change takes place which has nothing to do with reversal of the contracture. With weaker solutions the contracture may be sustained (F. B. Hofmann). After sufficiently weak solutions or sufficiently short exposures reversibility is good but it may be followed by a greatly delayed contracture ascribable to the sequelae of residual injury (Schwenker). The contracture is not propagated (Schenck) and is little affected by narcotics (Bethe, Fraenkel and Wilmers). The tension is somewhat greater than that produced by acids, amounting to 30 or 35 per cent of the tetanus value as compared with 15 per cent for acid (Bethe, 1923). Hydroxylamine and the alkylamines also produce contractures (Schwenker).

Narcotics. A similar two-phase contraction occurs with narcotics (Zenneck). All the narcotics behave in essentially the same manner, and the differences between them are rather of degree than kind. Chloroform is the one most studied but the alcohols and ether in higher concentrations produce similar phenomena (Kemp and Waller). Like alcohol in producing a contracture are benzine, xylol, carbon bisulphide, ethylchloride, petrolic ether and amyl nitrite (Herlitzka).

A chloroform contracture is initially reversible when the vapor is withdrawn (Tissot), but it is soon complicated by rigor. The tension is large (Verzár, Bögel and Szanyi), amounting approximately to that of a tetanus (Bethe, 1923). It thus cannot be an acid-contracture. The contracture is not conducted (Wilmers). It occurs in muscles rendered non-irritable to electrical stimuli by anesthetics and other means (Bethe, Fraenkel and Wilmers); and after extreme fatigue produced by an electrical tetanus—a condition in which the tension is about 15 per cent of maximum in spite of continued stimulation—chloroform raises the tension to normal (Bethe, 1923). In doing so it also produces rigor; but a reversible contracture of fatigued muscle can be produced

by alcohol (Kemp and Waller). The latter substance acts also on muscles anesthetized to electrical stimulation. Hin claimed that chloroform produces a contracture in a muscle caused to undergo spontaneous death and the passage through rigor in the absence of oxygen; but this could not be confirmed by Matsuoka.

Salts. Salt action itself is sufficient to produce a contracture. Wilmers found that 6 per cent NaCl and ten-fold Ringer's solution produced a contracture of the frog's sartorius; Frank, Nothmann and Gutmann observed that 5 cc. of either 10 per cent NaCl or 20 per cent glucose intra-arterially injected caused contracture of the denervated cat's tongue; and Laugier and Nouel obtained a greater contracture in frog-muscles electrically stimulated, when the muscles had been dehydrated by intraperitoneal injections of salts or sugars.

Of the cations potassium has aroused the greatest interest. Its action was shown by Grützner and by Zoethout, and the tensions it produces have been measured by Riesser and Richter and by Bethe and Franke. The former found a value of 20 per cent of the tetanus-tension in the frog's gastrocnemius, and the latter found a value of 35 per cent in the sartorius of the toad. Bethe and Franke also observed that the contracture is rapidly reversible but that high concentrations cause clouding of the muscle and secondary shortening. They further determined that the contracture occurs in anesthetized muscle and that rubidium and caesium resemble potassium, but are much weaker.

The anions have been arranged by Bethe and Franke in the order of the effectiveness of their potassium salts in producing the rapidly reversible phase of the potassium contracture. The order is

$$Cl < Br < \text{Salicylate} < I < NO_3 < CNS$$

with the position of SO_4 doubtful. As might be expected they follow the Hofmeister series. Contractures can also be produced by anions combined with relatively indifferent cations. Of the halides iodides are the most effective (Rossi 1911) but the bromides have some action (Schwenker). The sulphocyanates have received the most attention. As described by Worthington the contracture consists of a large primary phase followed by rigor. It is compared with chloroform by Rossi because of the reversibility of the response to low concentrations and the irreversible changes produced by strong solutions.

Calcium precipitants cause contracture. Sulphate and citrate contractures in the frog have been described by Wilmers, and a citrate contracture also occurs on intra-arterial injection in mammals (85).

Fibrillation is very marked with these substances and in the case of the oxalates and phosphates there is a sustained contraction of the nature of a tetanus, differing from a contracture in the fact of conduction (Wilmers). Wilmers' curves do not however exclude the possibility of some contracture, and a primary oxalate contracture has been described by Bottazzi.

Bile salts are particularly active. Bile itself has great stimulating power (Kühne 1860, p. 23), and its active fraction has been shown to be the salts. Sodium glycocholate is more active than the taurocholate (Schwenker). The contracture is readily reversible and according to Bethe develops a tension 30 to 35 per cent of that of a tetanus.

Caffeine produces a contracture which has been extensively studied. The contracture is slow in onset but eventually develops considerable tension. In the process secondary changes characteristic of rigor occur and the contracture is therefore completely reversible only in the early stages. In this respect it resembles the chloroform contracture. The caffeine contracture is always associated with definite histological changes; but these are not at all specific for caffeine as they are also produced by chloroform and by hypertonic sodium chloride. They are probably quite general in occurrence and of considerable importance, but unfortunately they await a useful interpretation. As described by Secher there is, even when as yet no shortening has occurred, a lateral shift of the fibrils which destroys the regular appearance of the cross-striations and causes the sarcolemma to wrinkle. These changes readily reverse on perfusion with Ringer's solution, but the higher concentrations destroy the cross-striations and cause the contents of the fibers to collect in amorphous clumps. The muscle becomes hard and white and goes into rigor.

A contracture resembling that caused by caffeine is produced by the sodium salt of monobromacetic acid. The action of this substance was discovered by Pohl and it has been further studied by Engel.

Other substances producing contracture are glycolaldehyde, methyl-iminazol, methyl glyoxal and the secretion of frog skin (Schwenker). In water rigor (vide Meigs) there is an accumulation of lactic acid, so it is possible that a contracture of the acid type may play a part.

II. Substances producing a contracture following a twitch. The type-substance of this group is veratrin. According to the conditions of the experiment and the state of the muscle the veratrin-twitches are very varied in appearance. In some there is simply a prolongation of the phase of relaxation, in others a second phase of

shortening appears which produces a hump following the twitch, and in still others there is a contraction so fused with the twitch as to give a single sustained shortening. Between these forms all gradations appear, the details of which are described in recent reviews by Boehm (1920) and Querido.

The reason why the veratrin-contracture only follows a twitch is still a matter of conjecture, but the properties of the process itself are quite well understood. In many respects it resembles the Tiegel contracture (Schenck, 1895). In the milder degrees of poisoning the intensity of the veratrin-effect depends upon the strength of the stimulus (v. Bezold and Hirt; Lamm, 1911); but in a well poisoned muscle every twitch is followed by a contracture even on indirect excitation. The contracture is very subject to fatigue. Repeated induction shocks cause the twitch to become normal but after a rest the veratrin-effect again returns. Whatever depresses the muscle causes the veratrin-contracture to disappear before the twitch (Lamm, 1912). When the poison is restricted to one half of the muscle the secondary contracture occurs in that end alone, no matter which end is stimulated (Biedermann, 1895 a). Ordinarily the tension is less than in the twitch proper, but in Buchanan's carefully done experiments tensions occurred in contractures of the fused type equivalent to a tetanus value. Bishop and Kendall have also found that formaldehyde can produce contracture-tensions about twice those of the initial twitch. Such tensions indicate that the mechanism responsible for the twitch is responsible for the contracture.

The veratrin-effect is not restricted to veratrin alone. It is produced according to Boehm by protoveratrin and aconitine; and Verzár and Felter have found that it is given very strikingly by formaldehyde and to a somewhat lesser degree by glyoxal, acetaldehyde, paraldehyde, acrolein and n-glycerinaldehyde. They have also collected from the literature instances of production of veratrin-like twitches by a large number of non-related substances: inorganic salts of Ba, Ca, Rb, Y, Na, Sr, Be and Cs (Brunton and Cash); $NaClO_4$ (Kerry and Rost); RbCl, CsCl, NaI, NaBr and butyric and propionic acids (Blumenthal); helleborein, digitalin, atropine, strychnine and caffeine (Bottazzi); and colchicin (Jacoby). To this may be added sodium oxalate and sodium citrate (Neuschlosz, 1922).

The foregoing substances differ in the intensity of their effect and in their power of producing contracture without a preceding twitch; but the fact that such varied chemical entities come into a common

group on the basis of their imitation of the action of veratrin indicates that they are revealing a fundamental characteristic of skeletal muscle. The property is not universal for all contracture-producing substances, however, for Verzár and Felter have found that the compounds listed in table 1 evoke nothing approaching it.

TABLE 1

ELECTROLYTES	ORGANIC ACIDS AND SALTS	ALCOHOLS	OTHER ORGANIC SUBTANCES
KOH NaOH	Lactic acid	Methyl	Chloroform
$FeSO_4$	Acetic acid	Ethyl	Urethane
$CuSO_4$	Oxalic acid	Propyl	Morphine
$ZnSO_4$	Butyric acid	Butyl	Toluol
HCl H_2SO_4 HNO_3	Tartaric acid	Amyl	Hydroquinone
KCN	Formic acid		Acetal
NaCNS	Caproic acid		
H_2PO_3	Sodium oxalate		

Some of these compounds have been found by other observers to produce positive results, and it therefore seems probable that whether or not a veratrin effect is obtained must depend somewhat upon the condition of the muscle and the procedure in the individual experiment.

Veratrin produces no visible change in a muscle except following a twitch, but most of the other substances can in addition act directly. Contractures in general are augmented by intercurrent twitches; and solutions too weak to act immediately are able to modify the nature of a twitch response. Caffeine is a good example of a substance having both actions. The form of the twitch curves may be seen in papers by Secher and Golowinski and these authors give references to the previous literature. Low concentrations of the drug (1/4000 (Secher)) produce an effect only when the muscle is stimulated; higher ones act directly as previously described. Also, a caffeine treated muscle subjected to a series of twitches develops a sustained contracture more readily than a resting muscle does.

III. SUBSTANCES PRODUCING A CONTRACTURE ONLY WHERE THE MUSCLE-FIBERS HAVE A SPECIAL SUSCEPTIBILITY. A special susceptibility occurs in the neural region of frog's muscle, in certain particularly differentiated muscle-bundles in the frog, in the red muscles of the fowl, and in mammalian muscle after degenerative section of the motor nerve.

Kühne (1860) and later Pollitzer (1886) demonstrated that at the two bands in the frog's sartorius, where according to Mays the nerve endings are densest, stimulation could be effected by weaker induction shocks than would excite the ends of the muscle. Then in 1907 K. Lucas, in examining this region with his slot electrode, observed a discontinuity in the curve connecting the strength and duration of stimulation. As the discontinuity was absent at the ends of the muscle, he associated it with some substance, which he called β, existing in the neural region only. All three observers noted that the greater irritability of the neural region persisted after the nerve ends had been blocked by curari and they thereby demonstrated that it must be due to some feature of the muscle fibers, themselves. Langley had postulated, because of the peculiar pharmacodynamic susceptibilities of the region, that this feature is a special receptive substance, probably of a chemical nature; and with this substance Lucas associated his β discontinuity. More recently Boeke (1921) has observed in the same region a typical histological picture which he describes as a periterminal network. This, too, is associated with the receptive substance. While both associations are widely favored, neither of them fits the situation without further assumptions, as both phenomena are obliterated by denervation (Watts, Boeke, 1921, p. 482). The greater irritability of the neural region as compared with the ends of the muscle, is not confined to electrical and certain forms of chemical stimuli, but holds, according to F. B. Hofmann and Blaas, for mechanical excitation as well. Furthermore Beritoff and Iaschwili have shown that this region has a greater power to shorten.

The principal substances depending upon the neural region for their action are nicotine and the quaternary bases. Also certain salts have been found to be much more stimulatory to this portion of the muscle. In view of the nature of the muscle fibers it is not at all surprising that such should be the case, and it seems quite probable that more chemical stimuli have differential thresholds of excitation at various parts of the muscle-fibers, than is at present realized.

Nicotine has been extensively studied by Langley (1905–1914). When a frog's sartorius is immersed in solutions of nicotine up to 0.1 per cent in strength, contracture occurs only in the neural region. Much greater concentrations (1 per cent) produce a contracture at the nerve-free ends but it is not clear that the contracture there is identical with that produced in the middle of the fibers. It is

smaller and not perfectly reversible, and the curves show the double hump characteristic of substances which also cause rigor. A drop of nicotine placed on the sartorius muscle causes a local contracture, and not once in a thousand observations did Langley see it conducted. However, in the more sensitive rectus abdominis he thought conduction took place independent of any possible spread of the solution. Nicotine also causes contraction of the red muscle of the fowl (Langley, 1905), but does not affect normal mammalian muscle.

Studies of the quaternary bases were first made by Boehm (1908), who found that frog muscles were caused to contract by cholin, muscarine (synthetic), neurine, valearin, and tetramethylammonium chloride. The great activity of acetylcholine was found by Riesser (1922). It works in solutions of 1/1,000,000 (1/50,000,000, on perfusion, according to Hess and Neergaard), and is particularly valuable as a laboratory agent, since muscles are not injured by it. The curve of contracture is smooth, showing no evidence of a secondary portion; and recovery is complete after long immersion in it, so that the same muscle may be treated with it repeatedly. When the muscle is loaded only by a light isotonic lever the contracture is maintained during the action of the drug. But if the curve be recorded isometrically it soon passes through a maximum. Even with an isotonic lever the nicotine curve passes through a maximum (Langley, 1913), as does also the curve produced by tetramethylammonium (C. R. Marshall).

Riesser made the interesting observation that only the upper one-fifth of the gastrocnemius of the frog is caused to contract by acetylcholine. An entirely satisfactory explanation of this fact is hardly found, however, in attributing the effect to the neural region, as Mays observed endings in the lower portion of the gastrocnemius, just as one would expect in a muscle having a penniform arrangement of its fibers. Light on this puzzle was afforded by Sommerkamp's discovery of specially differentiated portions of frog muscles. In the ileo-fibular muscle there is a portion whose fibers are more opaque than the rest and which can be isolated from them. When a piece so isolated is stimulated through its nerve it gives the same twitch as other parts of the muscle, but when stimulated directly by strong shocks it differs from the latter in the ease with which it goes into contracture. When stimulated by acetylcholine it develops a tension of 50 to 80 per cent of a tetanus, a value which is to be compared with 10 to 15 per cent in the gastrocnemius (Riesser and Richter) and 20 to 26 per cent in the rectus abdominis of the bullfrog (82).

We are thus left with the inferences that all neural regions are not alike and that certain muscle fibers have a special susceptibility.

Normal mammalian muscle does not respond to nicotine and the quaternary bases, but when sensitized by degeneration of the motor nerve it becomes very susceptible to their action. This was first shown for nicotine by Heidenhain and for acetylcholine by Frank, Nothmann and Hirsch-Kaufmann (1922). The action was also found by Dale and Gasser to be present in such other substances properly belonging to this group as cytisine, hordenine methiodide, tetramethylammonium chloride, nitrosocholine, and trimethylsulphonium iodide, all of which have nicotine-like qualities.

The sensitivity of amphibian muscle, which does not undergo a reaction of degeneration (Bremer and Gérard), is not increased by denervation, but nicotine and acetylcholine both act in the same manner as on a normal muscle (Langley, Simonson). It is this persistence of nicotine action after denervation as well as after the motor nerves are blocked by curari, that led Langley to consider the point of action to be some receptive substance in the muscle fibers themselves. Fowl muscle, like that of the frog, normally somewhat sensitive to acetylcholine, has its sensitiveness greatly augmented by denervation, and mammalian muscle, at a period so early after nerve-section that the full sensitiveness to acetylcholine has not developed responds like normal avian muscle (Gasser and Dale). The types of reaction thus shade into one another in a manner suggesting that they are fundamentally alike. Presumably, therefore, mammalian muscle normally inert develops a reactivity to the substances which are normally active in the neural region of frog and fowl muscle. When this occurs the muscle is very extensively involved, as it produces a tension of 80 to 90 per cent of the tetanus value and a tension-time curve which has the same general dimensions as a control tetanic fatigue curve (49). The tension is too great to be produced by any mechanism other than the one which is engaged by the tetanus.

The ions which have been associated with the neural region are K and CNS. The nicotine-like action of the sulphocyanates and their preference for the neural region was pointed out by Langley (1916), and an association between potassium and nicotine was also made by Burridge. Riesser (1922) observed that the upper part of the frog's gastrocnemius is more sensitive to potassium and he grouped the contractures produced by K, CNS, nicotine and acetylcholine under

the designation "stimulated contractures." But as will be seen later there is not on a stimulation basis enough difference between these and other substances to justify making a special group of them. All they have in common is an observed dependence on differentiated muscle, and this dependence is less in the case of the salts than it is for the organic substances. Both K and CNS act on the lower part of the frog's gastrocnemius (Neuschlosz, 1925b); and as already mentioned Rossi has called attention to the points of similarity in the action of CNS and chloroform.

Antagonisms and synergisms. The antagonisms, while interesting in themselves, add little to the knowledge of contractures. The general situation is revealed in table 2. Substances like calcium having a general action on the plasma membrane are generally antagonistic. Novocaine, which was shown by Neuschlosz (1922) to alter the physical-chemical properties of colloids, also has a widespread depressant action. In many contractures its mode of action may be secondary to colloidal alteration of some part of the muscle fibers as is supposed by E. Frank and Katz and by Riesser (1922). In other contractures, e.g., caffeine, the antagonism has been shown by Schüller to be chemical, novocaine like other local anesthetics and sodium salicylate forming an inactive complex with the stimulant. On the other hand in the sustained tetanic contractions resulting from tetanus toxin or decerebration, more susceptible structures seem to be involved; according to Liljestrand and Magnus, the proprioceptive endings which are responsible for the reflex drive of the muscle.

The other antagonists are not so general in their action. Curari has been very extensively studied by Langley (1905, 1914), particularly in connection with the stimulation by nicotine. He finds that the antagonism is only to the small doses which stimulate through the more irritable mechanism at the middle portion of the muscle-fibers of the frog's sartorius, and not to the large doses which stimulate the nerve-free ends. The amount of curari necessary depends upon the amount of the stimulant, an "equivalent percentage ratio" of curari to nicotine being necessary for each concentration of nicotine. The value of this ratio depends on the susceptibility of the muscle to the stimulant, being low $\frac{1}{50}$ for the sartorius and high $\frac{2.5}{1}$ for the rectus abdominis.

Nicotine stimulation and curari antagonism also occur in fowl muscle. After degeneration of the motor nerve the nicotine action

TABLE 2

	NOVOCAINE	CALCIUM	CURARI	ATROPINE	SCOPOLAMINE	ADRENALIN
Frog muscle						
Sodium hydroxide N/100	0[17]					
Potassium	0[20]	+[19,25]				
Ethyl alcohol (25–30 per cent)	0[17]					
Chloroform (1/3 saturated	+[17]		0[17]	0[17]		
Caffeine	+[20,23]			0[17]		
Quinine	+[20,23]					
Veratrin	+[20]	+[13,21]		+[20]		
Acetylcholine	+[20]	+[24]	+[20]	+[20]		0[10,20]
Nicotine	+[17,20]	+[14]	+[15]	+[17,20]		
Sulphocyanates	+[3,17,19]	+[19]		0[19]		
Tiegel's contracture				+[9]		
Galvanic current contracture			0[2]	0[2]	0[2]	
Repeated indirect stimulation (Bremer)			+[2]	+[2]	+[2]	0[2]
Mammalian muscle						
Tiegel's contracture				+[22]	+[22]	+[22]
Contractions: Decerebrate rigidity	+[16]		+[1]			
Contractions: Tetanus	+[18]		+[1]			
Denervated mammalian muscle						
Acetylcholine	+[5]	+[6]		0[5,6]	+[5,6] 0[12]	+[5,6,7]
Nicotine	+[5]		+[8]	0[5,7]	+[5]	+[5,7]
Potassium		+[6]			0[6]	+[6]
Lingual contracture of tongue			+[8]			+[4]
Dorsal root stimulation			+[12]			+[11]

[1] Bremer and Titeca.
[2] Bremer.
[3] DeBoer.
[4] Frank, Nothmann and Hirsch-Kaufmann, 1922.
[5] Frank, Nothmann and Hirsch-Kaufmann, 1923.
[6] Frank, Nothmann and Guttmann.
[7] Gasser and Dale.
[8] Heidenhain.
[9] Hess and Büsch.

is augmented, and, as would be expected, the curari antagonism decreases so that insufficient concentrations give the appearance of absence of antagonism, as in Edmunds and Roth's experiments. In denervated mammalian muscle curari, in dose sufficient to block the motor nerves, annuls the effect of nicotine on the tongue (Heidenhain), but not that of acetylcholine on the gastrocnemius (Gasser and Dale). However, judging from Langley's observations it is quite possible that a larger dose of curari in the latter case might be effective; and this supposition is borne out by the fact that in the frog, according to Boehm (1908) and Riesser (1922), greater concentrations of curari are necessary to antagonize nicotine and acetylcholine than are necessary for indirect block.

Langley associated the action of curari with his receptive substance because of the antagonism to nicotine, and Lucas found that curari also obliterated the β curve attributed to the same substance. The two sets of data are in accord with respect to the explanation of the various antagonisms. Indirect block, which Lucas found to occur when the β curve was little affected, must be due to an action on the nerve endings (Watts), or to the fact that a minute change in the receptive substance renders the stimulus from the endings inadequate. The action of nicotine on curarized muscle corresponds to the persistence of the β curve after indirect block (Lucas), and the antagonism of nicotine by greater concentrations of curari fits with the elimination of the β curve. The prevention of contracture from rapidly repeated stimuli applied through the motor nerve by doses of curari too small to produce indirect block, a fact observed by

[10] Hess and Neergaard.
[11] Hinsey and Gasser.
[12] Hinsey (1928).
[13] Lamm (1912).
[14] Langley (1909).
[15] Langley (1914).
[16] Liljestrand and Magnus.
[17] Matsuoka.
[18] Meyer and Weiler.
[19] Neuschlosz (1925).
[20] Riesser (1922–23).
[21] Rossi (1911).
[22] Schäffer (1920).
[23] Schüller and Athmer.
[24] Simonson.
[25] Zoethout.

Bremer and Titeca, is logically attributed by the authors to a Wedenski effect. The concentration of curari needed in each case seems to be dependent on the effectiveness of the stimulating agent.

There is one serious objection to the above formulation. The β curve has not been found in denervated muscle (Watts), as should be the case in view of the persistence or augmentation of the action of nicotine after denervation. Lucas' data show that there is some probability of missing a β curve, even if it be present; and unless this be the explanation of its absence in denervated muscle, the interpretation of the relation of the β curve to the receptive substance will have to be abandoned, or the assumption made that the receptive substance is so altered by denervation that it does not show in a chronaxie curve although it retains its drug sensitiveness. Although the greater irritability of the neural region to electrical stimulation has not been found to survive degenerative denervations, it is known to persist for at least one other form of stimulation: namely, mechanical stimulation (F. B. Hofmann and Blaas).

Adrenalin does not modify the contractures of frog muscle but Frank, Nothmann and Hirsch-Kaufmann (1923) made the interesting observation that it prevents the contractures of denervated mammalian muscle. The antagonism applies both to the contractures produced by chemical stimulation and to those resulting from stimulation of the dorsal roots (116); and it is certainly not entirely secondary to vascular changes (86). In Schäffer's experiments on the Tiegel contracture in man, the response to strong electrical stimulation was also decreased by adrenalin. Thus, a substance which apparently increases the power of a fatigued muscle to produce twitches (Gruber), decreases its ability to undergo a sustained contracture. A somewhat similar case has been found by Bishop and Kendall. Histamine, which opposes the action of formaldehyde on the retractor penis of the turtle, tends to make the twitches higher and faster. How this occurs has not been investigated, but it is perhaps of some significance that Meyerhof and Lohmann (1926) have shown that a series of twitches is greater in height when produced by electrical stimuli too weak to cause a contracture. The antagonism by adrenalin of the stimulatory action of certain drugs could be explained as a prevention of fixation of the drug on the muscle surfaces, but such an explanation would not apply to Tiegel's contracture.

Atropine prevents some of the frog contractures but is ineffective against the contractures of denervated mammalian muscle. Massive

doses of scopolamine are antagonistic to the latter (Frank, Nothmann and Hirsch-Kaufmann, 1922) but ordinary doses are quite without action (Hinsey, 1928).

Synergisms. The action of acetylcholine and similar drugs on denervated mammalian muscle is potentiated by physostigmine (Nitschke; Gasser and Dale) and by guanidine (Frank, Nothmann and Guttmann), although neither of these substances is alone able to induce a contracture. Pilocarpine and physostigmine according to Schäffer also increase a Tiegel contracture in normal human muscle. The synergistic power of these drugs must be due to their stimulant action. The power of physostigmine to produce fibrillation is well known, and in the fowl Edmunds and Roth have shown that it can bring about a low grade contracture. The substance apparently augments the excitability of the irritable membranes in denervated muscle so that the exciting substances are more effective.

Concerning the mode of action of atropine, physostigmine and adrenalin on skeletal muscle, little is known beyond the fact that one action is on some part of the muscle itself, since the drugs are still effective after degenerative section of the motor nerve. The only receptive substance in muscle, which there is reason to believe is connected with an innervation, is the one associated with the motor nerve; but from the pharmacological properties of the muscle stimulants on other tissues the assumption of an autonomic innervation has several times been made. E. Frank (1920) has assumed a parasympathetic innervation, largely on account of the parasympathomimetic qualities of acetylcholine; and this view has been supported by Riesser (1921) as the result of his observations on frog muscle; also, Schäffer (1920a) has assumed in addition an inhibitory sympathetic innervation, on account of the adrenalin antagonism.

There are two major objections to the above assumptions. Dale and Gasser have shown that the common property of the stimulatory bases is their nicotine-like action, and that conspicuous parasympathetic stimulants like true muscarine, arecoline and pilocarpine, which lack this action, are inert. In the second place the assumption of an innervation of a tissue from its response to drugs is not justified (Schilf, p. 77). The relationship of innervation to drug susceptibility is not sufficiently precise. We need only to cite the well known case of the pilocarpine stimulation of the sympathetically innervated sweat glands and the case found by Luckhardt and Carlson in the turtle, where adrenalin constricts the lung vessels which receive their

innervation from the vagus. The classical formulations of the relation of drug action to innervation are far too rigid; and to bring theory more into accord with the facts Langley (1921, 43) expresses the view that the action of poisons is due to a differentiation of tissue cells in the course of phylogenetic development, wherein receptive substances or chemical groupings are formed independent of the innervation. The action of the innervation would thus be determined by the terminal mechanism with which it makes connection and the action of a drug might take place where there is no innervation at all.

If the assumption of an innervation from a drug stimulation be unwarranted, the assumption of one from an antagonism is even more so (Magnus, 1908). For instance atropine antagonizes such varied responses as the barium contraction of the intestine (Roth), the adrenalin contraction of the spleen (Hoet), the contraction of a sensitized guinea-pig uterus in the presence of the antigen (Stoland and Sherwood), the action of veratrin on skeletal muscle (Riesser 1921, 1922), Tiegel's contracture (Hess and Büsch), and the sympathetic secretion of the submaxillary gland (Langley, 1878). Atropine thus has a widespread depressant action on stimulations from various causes, and whether or not an "antagonism" can be demonstrated depends upon the relative effectiveness and dosage of the stimulant and of the atropine.

METABOLISM IN CONTRACTURES

A review of the metabolism in contractures must be prefaced by a preliminary note as to the present status of the knowledge of the metabolism occurring in tetanic contraction. The problem must be considered with the possible sources of energy for muscular activity clearly in mind.

Little need be said concerning the relationship of lactic acid production to the energy liberation in muscular contraction. The beautiful classic experiments of A. V. Hill and of Meyerhof, which established this relationship, are now described in every modern text book of physiology. A note as to the phosphate metabolism is, however, necessary. A recent important contribution to the biochemistry of muscle has been the finding by Fiske and Subbarow and by Eggleton and Eggleton of a labile phosphorus component. This the former showed to be a creatine phosphate compound. It was called "phosphagen" by the latter. It is broken down independently of carbo-

hydrates during contraction and is restored aerobically during recovery.[1]

The energetics of phosphagen was studied by Meyerhof and Lohmann (1927). They found that the cleavage of creatine phosphate is attended by a considerable liberation of energy, and this fact at once suggested that the substance might contribute to the heat production in muscular contraction. They therefore suggested a revision of the earlier balance sheet, in which all the heat, liberated at the same time as the production of unit quantity of lactic acid, was accounted for by heat-producing processes connected with lactic acid itself. The heat yield from the cleavage of creatine phosphate they determined to be 150 cal. per gram. The ratio of the PO_4 liberated to the lactic acid formed was found to vary between 1.5 and 0.75, with a mean of 0.8 to 0.9. This would account for about 125 cal.; 185 calories as in previous evaluations were attributed to the difference between the heat values of hydrated glycogen and dissolved lactic acid. This left 80 cal. to be explained, as the total heat to be accounted for, or the amount liberated at the same time as 1 gram of lactic acid, is 390 cal. This difference was made up by the heat that would be liberated if neutralization of the lactic acid were effected by equal amounts of protein and phosphate.

A modification of the above tentative formulation was soon necessary on account of a reinvestigation by Meyerhof and Lohmann (1928b) of the heat yield from phosphagen. It was now found to be 110 to 120 cal. per gram instead of 150; and during the period of measurement the ratio of PO_4 to lactic acid was observed to be on the average about 0.5. But this modification still permitted the possibility that phosphagen might be an important source of energy, particularly in a twitch or early in a tetanus when the ratio of PO_4 to lactic acid would be higher than 0.5. However as the problem unfolded, new data cast grave doubts on such a view.

Meyerhof and Lohmann discovered that in addition to the aerobic restoration there is an anaerobic one. This was found in a later investigation by Nachmansohn to amount to one-third of the phos-

[1] The following paragraphs were written in the latter part of 1928. During the period that this review has been in galley form the chemistry of the phosphates in muscle has made considerable progress; but nothing has appeared to alter the conclusion drawn as to the relation of the phosphate metabolism to energy liberation. The chemistry of the phosphates has been reviewed in the REVIEWS by Eggleton (1929) and additions to the subject have since been made.

phagen broken down in normal muscle and to be completed in twenty seconds. As a resynthesis would be endothermic and demand another energy yielding process of a nature not determined, Nachmansohn suggested that the resynthesis is in effect only a restabilization. One-third of the phosphagen metabolized would thus be eliminated as a source of energy; but there would still remain the other two-thirds, which must be oxidatively restored. Doubt concerning this fraction first arose as the result of another interesting observation of Nachmansohn's. He found that curari, in amounts sufficient to block indirect excitation, greatly inhibited the cleavage of phosphagen; nevertheless the same tension per milligram of lactic acid could be obtained as from a control normal muscle in which there would be a greater phosphagen breakdown. A similar though more intense depression of the phosphagen metabolism was found by Meyerhof and Nachmansohn in muscles whose nerves had been blocked by tetramethyl-ammoniumchloride. In the paper in which this was reported the authors made the remark that the views regarding phosphagen as a source of energy would have to be modified.

In the meantime A. V. Hill (1928) had performed some new experiments on the relation of heat to tension, which demonstrated that in good muscles the ratio Tl/H (l is the muscle length) remains constant even when very considerable fatigue has supervened. Starting from the premise that the isometric coefficient of lactic acid remains constant up to a fair degree of activity he argued that, since the major portion of the phosphagen is broken down in the early stages of a long series of twitches, the ratio Tl/H should rise as the rate of phosphagen breakdown falls off. But his experiments had shown that this is definitely not the case; the ratio is constant. So he suggested either that purified phosphagen must have different thermal properties from the substance existing in living muscle or that the breakdown does not really occur. The alternative to such a "breakdown" was conceived to be some kind of an "unstabilization" which would permit the phosphagen to be broken down by the chemical treatment necessary for its estimation. This assumption apparently differs from Nachmansohn's in that it would involve all the phosphagen and not merely the fraction anaerobically resynthesized.

Thus whatever may be the function of phosphagen in muscle physiology, it cannot at the present time seriously be considered as a a source of energy (Meyerhof and Nachmansohn suggest that it is

associated with excitation rather than with contraction proper). Lactic acid again dominates the field but how it is involved is a matter of theory only. Controversies arise on this subject, but fortunately for our purposes it makes little difference whether lactic acid is involved as an acid or is merely a relatively stable end-product of a series of chemical reactions; the fact still remains that it is a signal to the experimenter that a given reaction, yielding a large amount of energy for muscular contraction, has taken place. Examinations of contractures for the production of lactic acid therefore are of preëminent importance for the identification of the mechanism involved in the muscle.

Lactic acid. The intensity of the lactic acid metabolism can only be judged from determinations of lactic acid itself. Many inferences have been drawn from estimations of the lactacidogen of Embden in the belief that, since it breaks down into free phosphate and lactic acid, the decrease in lactacidogen measures the increase in lactic acid. But these inferences cannot hold, because they fail to recognize the possibility of a phosphate synthesis during a tetanus (or contracture). Embden and Lawaczeck found that tetanized muscles thrust into liquid air contained less free phosphate (the free phosphate here included phosphagen) than fresh muscles or muscles at the beginning of a tetanus, treated in an identical way: an observation which they interpreted as meaning a resynthesis of lactacidogen assumed to be split as the result of the cold stimulus, or the cold stimulus plus a short tetanus. Although the experiments do not clearly reveal how much, if any, of phosphate ester was broken down by the short initial stimulation, they do show a phosphate synthesis during the tetanus; and the existence of such a synthesis is borne out by the observations of Eggleton and Eggleton and Irving and Bastedo, who observed that the rise in inorganic phosphate in an active muscle will not account for the phosphocreatine decomposed. The latter suggest that the phosphate is synthesized into lactacidogen. In extreme fatigue and rigor the free phosphate again increases, but the situation is now further complicated by the assertion of Lohmann (1928) that this increase in free phosphate can be accounted for to the extent of 75 to 90 per cent, as coming from pyrophosphate, whose breakdown is quite independent of lactic acid production. Lohmann further claims that the natural preformed phosphorus compounds are but little affected in rigor, although the hexosemonophosphoric acid ester of Embden, when added to a muscle hash, is completely split.

On account of this varied and at present somewhat uncertain behavior of the phosphates it is not surprising that lactic acid determinations do not coincide with inferences from lactacidogen determinations. Laquer's data for fatigue show a lactic acid increase with no increase of free phosphate. In ammonia contractures,while the lactic acid is increased, so is the lactacidogen according to Riesser and Heianzan; and the same is true for the contracture produced by the sodium salt of monobromacetic acid (compare the lactic acid determinations of Engel with the lactacidogen determinations of Schwartz and Oschmann).

Our knowledge of the production of lactic acid in Tiegel's contracture is derived from experiments performed for the purpose of ascertaining the duration of lactic acid formation in relation to a period of tetanic stimulation. Embden and his co-workers (1925) observed that, over periods of 10 to 30 seconds following a ten-second tetanus, additional lactic acid was produced in amounts corresponding to as much as 40 per cent of that formed during the tetanus. On account of the theoretical significance of this observation in the problem of contraction, the matter was reinvestigated by Meyerhof and Lohmann (1925; 1926) and by Suranyi (1926). They found that the extra lactic acid did not appear when a muscle was stimulated indirectly or directly with just maximal shocks, but as much as 90 per cent extra lactic acid developed after supramaximal direct stimuli. Tiegel's contracture rarely occurs after indirect stimuli, but is readily produced by strong direct stimuli. It shows very beautifully in Meyerhof and Lohmann's curve 107 (1926) made with direct supramaximal induction shocks two seconds apart, in an experiment in which there was an extra production of lactic acid, as compared with a control muscle stimulated at the same rate with normal shocks. In figure 3 of Suranyi's paper the tension curve from a supramaximal tetanus reveals a persistence of tension for some seconds after cessation of the stimulation; and the amount of the extra lactic acid found is in relation to the amount of contracture.

That lactic acid is formed in fatigue contractures, rigor mortis and thermal contractures was known to Ranke (1865). Its presence was placed on an unequivocal basis by Fletcher and Hopkins (1907) and their experiments have been abundantly confirmed. In the chemical contractures lactic acid has been found in most of the instances in which a search has been made for it. It is produced by the anesthetics: alcohol, chloroform, urethane, and chloral hydrate (Fletcher

and Hopkins (1907), Meyerhof (1921)); by the lower fatty acids (Meyerhof, 1924); by alkalies: $NaHCO_3$ (Laquer, 1914), NaOH (Meyerhof, 1924), and ammonia (Zondek and Matakas); by caffeine (Ransom, Meyerhof and Himwich, Matsuoka); by the sodium salt of monobromacetic acid (Engel); by sulphocyanates (Riesser and Richter); by nicotine (Matsuoka); by KCl or quinine (Zondek and Matakas); by acetylcholine or trimethylsulphonium iodide, acting on denervated mammalian muscle (Gasser and Dale); and by acetylcholine acting on normal fowl's muscle (ibid.).

The one case that has resisted the demonstration of lactic acid formation is the acetylcholine contracture in frog muscle. Both Riesser (1922) and Meyerhof (1924) make the statement that no lactic acid is formed but neither publish any data. Zondek and Matakas using the frog's gastrocnemius cite four experiments, in two of which the lactic acid was greater than in the controls, and in two less. In some preliminary experiments in this laboratory on the rectus abdominis of the bullfrog, data were obtained very much resembling those of Zondek and Matakas. Some stimulated muscles contained more lactic acid than the controls and some less, with differences greater than in paired untreated muscles. It must be admitted that the experiments to date afford no evidence for lactic acid production, but it is equally true that it is impossible to conclude that none is formed. The problem must be considered unsolved; but it demands a solution inasmuch as the rectus abdominis muscle develops a tension one-quarter of that in a tetanus, and the source of the energy for this tension is unknown. Lactic acid is produced in normal fowl's muscle and denervated mammalian muscle stimulated with acetylcholine, and it is hard to believe that it is not formed in the frog's contracture which is so closely analogous. It is particularly difficult to see where Sommerkamp's muscle could obtain the energy for a tension 60 per cent of that obtaining in a tetanus, except from the lactic acid metabolism.

In spite of the numerous demonstrations of lactic acid production in the contractures, its connection with them has not gone unchallenged. Objections have been raised for a number of reasons.

Verzár (1922) and Bethe and his co-workers take the stand that lactic acid cannot in any case be the cause of muscle contraction, and that it is rather to be considered the end product of a metabolism than the active agent. The opinion is based on the low tensions of acid contractures as compared with tetani; but all this fact really

proves is that lactic acid produced from the muscle's energy stores cannot act like acid added from without. The objection is fully met by Meyerhof and Matsuoka, who reiterate that in the normal contraction lactic acid must be formed at a sufficient *velocity*, and in the appropriate positions in the muscle.

The rôle of lactic acid in the muscle mechanism is not of great importance from the standpoint of the contractures; but another contention of Bethe's, i.e., that the contractures produced by heat, chloroform, acids, alkalies, potassium, bile, etc., do not involve the lactic acid mechanism at all, is of immediate concern. Bethe evolved the theory that these substances act directly upon some contractile structure in the muscle, largely to account for the tensions larger than those produceable by acids, which may be developed in muscles rendered non-irritable by narcotics and other means. The irritability problem will be discussed in another section, but the relation of the theory to muscle metabolism will be considered at this point.

Several considerations suggest the improbability of direct chemical action. It is unlikely that such highly heterogeneous chemical substances as are involved would act directly in the same way, and one would hardly expect the large amounts of energy known to be liberated not to be utilized. Also, Meyerhof (1924) points out that a rapidly diffusible substance like chloroform, if acting directly, should produce its full action at once, whereas actually the chloroform contracture is slow in onset. But there is a more serious objection to the theory, in that if the substances act directly and the lactic acid energy be waste, the lactic acid production and tension development should be independent variables. This is not the case. Meyerhof and Himwich found that muscles obtained from fat-fed rats and therefore containing but little carbohydrate, produced very little tension and very little lactic acid in the contractures from chloroform, caffeine, and heat, as compared with muscles from rats on a normal dietary. And Matsuoka showed that in caffeine and chloroform contractures in the frog, temperature variations in the rate of lactic acid liberation were accompanied by comparable variations in the rate of tension development. The lactic increased in the muscles throughout the period of rising tension, but stopped increasing when the tension reached a stationary state.

If the isometric tension records of chloroform, caffeine, and other contractures be examined, it is apparent that they always run

through a maximum instead of approaching the steady state asymptotically. This suggests that the *rate* of lactic acid formation is in some way connected with the tension. The chemical data are not sufficient for the plotting of the curve of lactic acid production; but the cessation of lactic acid formation, when rigor tension is reached, indicates that the curve is decremental; and this supposition is fully supported by the curves of heat production made by A. V. Hill (1912) and by Peters, from data obtained in experiments performed with a microcalorimeter. In these experiments, presumably due to the high concentrations of chloroform employed the induction is so rapid that the rate of heat production is maximal at the start. However, in the caffeine experiments made by Hartree and Hill (1924) with a thermopyle, the rate clearly passes through a maximum.

A position similar to Bethe's is taken by Zondek and Matakas, who dispose of lactic acid by saying that it is formed only *after* the contracture, and therefore is due to injury. They base their opinion on experiments in which the lactic acid determinations, made immediately after the onset of contracture, were negative. But to argue that no lactic acid was there is like denying its production in a single twitch because it cannot be chemically determined. Just as lactic acid is soon demonstrable in a series of twitches, it was soon demonstrable in their contractures produced with KCl, quinine, ammonia, and chloroform; and in the case of the last three substances it was present in abundance before the height of the shortening. The time of maximum intensity of action was not measured with a tension lever.

Ritchie (1924) perfused a muscle first with neutral red and then with Ringer's solution saturated with chloroform, and found that contracture set in previous to any change in the reaction of the muscle. From this he concluded that the immediate effect of chloroform cannot be due to acid. This conclusion is open to the same objection as that raised against the opinion of Zondek and Matakas; chloroform acting as a stimulant could mobilize acid with the production of contracture long before sufficient acid would diffuse out into the sarcoplasm to turn an indicator. In fact the situation in chloroform contracture is shown by this experiment to be identical with that occurring in a tetanus; for in tetanic contractions the failure of a muscle, which has undergone moderate activity, to change in reaction has been shown by Pechstein from electrotitrations of muscle juice, by Ritchie (1922), himself, using a MnO_2 electrode in the

muscle, and by Verzár (1922) employing indicators. It is therefore curious that Ritchie should conclude in the case of a tetanus that the lactic acid concerned in the contractile process can be in the free state only momentarily, and then, as the result of what is fundamentally the same experiment, deny a connection between acid and chloroform contractures. Clearly in making the two judgments he did not have in mind the probable similarity in the nature of the two processes, but conceived the chloroform contracture to be simply an acid-contracture.

The sum of the evidence indicates that at least the greater portion of the chemically induced contractures are not acid-contractures (vide also Meyerhof, 1924). In many cases the tensions are too high, in others it has been shown that the contracture starts before there is any appreciable acid accumulation. The contractures must be produced by energy liberated from the same sources tapped in a tetanus. There is only one alternative, direct action of the chemical substances, a view which is improbable for reasons already stated.

The contractures of extreme fatigue and rigor mortis. The view that these are due to the action of the accumulated acid is widely held because of their many points of similarity to acid-contractures. According to Bethe (1923) the maximal tensions in fatigue contractures are the same as those produced by acid added from without, and the concentrations of acid necessary in the two cases are comparable. He finds the minimum concentration of HCl *in* the muscle for the production of maximal tension to be 0.02 to 0.04 N; and the latest figures of Meyerhof and Lohmann (1926) for the lactic acid concentrations in fatigued muscles are, depending upon the conditions of the experiment, 0.022 to 0.05 N. Prevention of acid accumulation in a dying muscle prevents the rigor contracture. This was accomplished by Winterstein who washed the muscle with Ringer's solution so that the lactic acid could diffuse away.

There are but two possibilities as to the origin of a fatigue-contracture. The acid must act either directly or through the normal mechanism,—and for the latter alternative there is little evidence. The general effect of acids as shown by Kura Kondo and others is to decrease lactic acid formation from other causes. Whether or not acids mobilize lactic acid at all in an irritable intact muscle seems to have been little studied. The only note on this point is by Meyerhof (1924), to the effect that the lactic acid content of a muscle is increased by the lower fatty acids. That there is plenty

of available energy at the time of a fatigue contracture follows from the difference found by Fletcher and Hopkins between the lactic acid content of muscle in fatigue and in chloroform rigor; but there is no evidence that it is being used. The same experiments demonstrate that from the standpoint of energetics the increase of tension which chloroform produces in fatigued muscle is readily accounted for without the assumption of a direct action of the chloroform.

The theory that the tension production in rigor mortis is due to an acid-contracture is totally unable to explain the experiments of Hoet and Marks. The authors do not consider that the rigor which they observed is entirely different from the one occurring in normal muscles; but the phenomenon as they describe it is so clearly not an acid-rigor, that either some process other than the effect of accumulated acid must occur in the rigor mortis of normal muscles, or their rigor is a different event. What Hoet and Marks found is that in conditions where the muscles are essentially glycogen-free, conditions which may be produced experimentally either by thyroid feeding or insulin (Baur, Kuhn and Wacker), rigor occurs almost instantly after death without the development of acidity or the accumulation of lactate ions. The rigor has some peculiarities, however. It occurs with less than normal tension, passes off more rapidly, and if broken by force it is not reëstablished.

The initial contracture could be explained without any difficulty if we assume a trace of residual glycogen, since from the analogy with a twitch or the earliest stages of a chloroform contracture, demonstrable amounts of lactic acid would not be necessary for the assumption of lactic acid action. As the authors state, the absence of glycogen would explain the weakness of the contracture, its short duration, the failure to redevelop when broken by force, and the absence of lactic acid accumulation; but there still would remain the problem of how the contracture could persist at all in the presence of as little lactic acid as the data would permit us to assume. Maintenance energy, such as evolved in a tetanus, could not be derived from carbohydrate and there is no acid to act directly. Hoet and Marks, finding the lactacidogen content of the muscles to be low, attribute the rigor to failure of phosphate "resynthesis" due to want of carbohydrate. But they leave as an open question how such a failure could produce rigor, and the more recent knowledge of the phosphates is not helpful. The most convenient type of theory from which to derive an explanation would be one postulating the

failure of a relaxation process. The contracture would then be as A. V. Hill (1926) suggests an "unreversed contraction."

Phosphate metabolism. Of the phosphagen metabolism nothing is known; but changes in lactacidogen have been found. In contractures produced by acetylcholine, potassium, or sulphocyanates no change takes place (Riesser, 1921; Neuschlosz, 1927). In caffeine and chloroform contractures lactacidogen decreases (Riesser and Neuschlosz, 1922); in ammonia and monobromacetic acid contractures it first increases, then decreases (Engel, Schwartz and Oschmann, Riesser and Heianzan). It is probable that the usual course of events is an increase of lactacidogen followed by a decrease, but that the first stage is passed over in the contractures from the more intensely acting chemical substances. The interpretation of these experiments awaits the final solution of the phosphate problem.

HEAT PRODUCTION IN CONTRACTURES. Myothermic experiments have made their greatest contribution to the knowledge of the contractures by establishing the nature of those of the veratrin-type. Starting from the conception that the contraction of a muscle is mediated by some substance formed at the time of stimulation, Fick and Boehm in 1872 argued that the effect of veratrin would be either to cause this substance to be liberated in greater amount or to delay the process of restitution. In the first case the temperature would increase more than in a normal twitch, and in the second case less, because the heat producing processes would be distributed over a longer period. With the problem thus posed they proceeded to measure the heat and they found it to be much greater than normal. Thus, in what is a model of clear thinking followed by experimentation, the problem was solved. The experiment has recently been repeated by Hartree and A. V. Hill (1922) with their greatly improved technique, in which not only the amount of heat produced could be measured but its time course ascertained. They not only confirmed the increased production of heat, but established what Fick surmised but could not prove: namely, that the production is proportional throughout the twitch to the force maintained. The evidence is, therefore, overwhelming that the veratrin-twitch is due to an increased liberation of energy; and it is indeed surprising that so many authors still speak of it as a delayed relaxation.

In the case of caffeine, Hartree and A. V. Hill (1924) found a very similar situation. Muscles stimulated after treatment with dilute solutions developed more heat than normal muscles, but the increase

was not as great as that produced by veratrin. Strong solutions which produced a spontaneous contracture, caused a spontaneous liberation of heat lasting until as much energy had been released as could be mobilized under the most ideal conditions by electrical stimulation to complete exhaustion. It was thereby shown that the action of caffeine is to liberate slowly and continuously the chemical processes normally released by stimulation.

In Tiegel's contracture an increased heat production was to be expected from its similarity to the veratrin-effect, and from the heat production which Fick observed in the analogous condition produced by a constant current (personal communication to Schenck, 1895). Meyerhof and Lohmann's observations (1925) of the continued production of lactic acid after cessation of direct stimulation with supramaximal induction shocks increased the expectation; and the heat was actually found by Furusawa and Hartree in amounts commensurate with the lactic acid formed. As recently described by Hartree and A. V. Hill (1928) this heat starts out at a high rate at the end of a tetanus and falls rapidly to a low value, being complete in a minute or two. By it what is known as the "delayed anaerobic heat" is explained.

Of other forms of contracture few thermal studies have been made. Neergaard (1924) studied the acetylcholine contracture in a frog and found a heat liberation of the order of that occurring in a 2 to 3 second tetanus. No tension records were made; and as the metabolism in this contracture is unknown no check is possible between various forms of data. Considering the low tensions in acetylcholine contractures in the frog and the evidence for a very low metabolism, the heat values found seem high. In any case no conclusion can be drawn until the subject is further developed.

ELECTRICAL POTENTIALS IN CONTRACTURES. The number of investigations in this field is not large and the meaning of many of the data obtained is obscured by doubts as to whether the potentials as recorded are faithful representations of the potentials as they exist. Also, in many instances considerable uncertainty prevails as to the sources of the potentials observed. The technical question at issue is whether the action potentials in the contractured muscle are smooth or oscillatory. With a slow recording instrument high frequency oscillations will be fused and give the appearance of a smooth curve; therefore, in every case where smooth curves are obtained the technique must be considered, to ascertain the probability of oscillations

being recorded if present. On the other hand if the recording mechanism be too sensitive adventitious waves may occur. A trained electro-physiologist is usually able to recognize these in his own records but it is not always so easy to do so in those of other observers.

Twitch potentials may be recorded monophasically or diphasically; but diphasic leads are not as a rule very satisfactory for contractures. The slow time-course of their potentials and the absence of wave-like transmission tends to make the leads isoelectric; then the recorded potential differences are no longer an index to the state of activity of the muscle, and such differences as do appear are very dependent upon structural peculiarities. A much clearer interpretation is possible if the potential under one electrode be made as nearly constant as possible by killing the muscle. The potential at the active electrode is then compared with an inactive point of reference.

Description of the electromyograms. The first contractures which will be considered are those of the veratrin-type, in which the electrical response is composed of the wave properly belonging to a twitch followed by a second potential change associated with the contracture. Historically the veratrin electromyogram was the first to be investigated; but inasmuch as there has been some difference of opinion as to the nature of the response to this particular drug, the potential changes produced by veratrin can best be understood in the light of the electromyogram of another contracture belonging to the same group, that produced by formaldehyde. In muscles poisoned by this drug or by glycerine, Verzàr and Peter found that the contracture following a twitch was accompanied throughout by a potential change which was entirely free from oscillations. Since the presence or absence of oscillations is of the greatest importance from the standpoint of interpretation, it is well that the smoothness of the electromyogram in the formaldehyde contracture has been confirmed by Bishop and Kendall with the aid of the Braun tube oscillograph, an instrument whose ability to follow small rapid oscillations is unquestionable.

Though the veratrin-contracture was often spoken of as a tetanus by the older investigators, Schenck (1896), Burton Sanderson, and Garten all found that the action current curve was continuous. Then P. Hoffmann (1912), using the string galvanometer, observed that the veratrin-effect was accompanied by an oscillatory action current whenever it was produced by a weak solution, but was wave-

free when produced by a strong solution. With the same instrument, but with a monophasic lead, DeBoer (1913) found oscillations only in the early part of the curve, the latter part being smooth. Some curves were smooth throughout. More recently Querido has found oscillations with all strengths of veratrin, and he attributes DeBoer's results to too tight a string. It does not necessarily follow, however, that this was the case. In fact there is less difference between the records published by the three observers than there is between their interpretations. In every case the oscillations get smaller as the contracture progresses. This is explained by P. Hoffmann, who defends the theory that the veratrin-effect is tetanic, on the assumption that the waves become so numerous as to be fused. The fusion theory, however, has not passed without criticism, for recently Dittler and Freudenberg have raised the objection that the transition from the oscillatory to the non-oscillatory phase should be attended by a demonstrable preliminary doubling or tripling of the rhythm, and that this according to Hoffman's curves is not the case.

From the mere inspection of an electrical record, in which there is a constant deviation with superimposed wavelets, it is impossible to decide whether the tracing is produced entirely by a fusion of individual waves, or whether it is caused by a continuous potential with superimposed oscillations. The former alternative is assumed in the interpretation of the veratrin-effect as a tetanus; but it is equally possible in analogy with observations on other chemically induced after-contractures to assume a continuous element in the action current. In the latter case the oscillations, of whose existence there can be no doubt, would have to be attributed to an *added* feature of the nature of a tetanus; but this feature can readily be supplied in the fibrillary twitches which Lamm (1911) has described as occurring during the contracture. The fibrillations, which are present to a variable degree in different instances, are beautifully illustrated in Lamm's curves. It would be interesting to know whether in a muscle poisoned at one end only the two potentials postulated might not be separated by the propagation of one of them and the failure of propagation of the other.

The locus of an ideomuscular contracture was found by Biedermann (p. 332) to be negative to a normal portion of the muscle. Alcohol contractures were studied by Kemp and Waller. A negative variation accompanied the mechanical response and disappeared when the muscle relaxed on washing.

The contracture of frog's muscle, following a series of twitches, was examined by Beritoff and Woronzow with a string galvanometer capable of following 700 vibrations per second. When employing a monophasic lead they found that the active electrode maintained a perfectly oscillation-free negativity throughout the contracture. The stimulation of the muscle was indirect, so the contracture was probably largely a contraction remainder, though it is not clear from the description that Tiegel's contracture was altogether excluded. No differentiation between these two forms was considered in the paper.

In the few cases where electrical studies have been made of chemical contractures occurring spontaneously, the potential curves have been continuous. E. Fischer found a continuous potential to obtain in the contractures produced by K, NaOH, HCl, and chloroform. In the case of the last mentioned substance the finding was in confirmation of Herlitzka.

The acetylcholine contracture in the frog is attended, according to Riesser and Steinhausen, by a smooth deflection of the galvanometer string. Schäffer and Licht found the same to obtain in denervated mammalian muscle, and they also observed it in perfused frog muscle, in which condition it had been missed by v. Neergaard (1924). When fibrillations were present they were revealed by oscillations superimposed on the constant potential.

Views as to the sources of the potentials. According to the prevailing opinion, the electromyogram is attributed to a wave of activity which spreads along a muscle fiber and reveals itself at any small segment by producing a very transient fall of potential (with respect to resting tissue) at a time immediately preceding and perhaps extending slightly into the period of muscle shortening. In a tetanus these waves are repeated in rapid succession. Therefore those who with P. Hofmann believe in the tetanic nature of some of the responses spoken of as contractures, have a secure foundation for the interpretation of the oscillatory or fused electromyogram in the known properties of excited muscle.

On the other hand those who have found non-oscillatory electromyograms, in the want of any muscle potential with which to associate them, have generally interpreted them in ways not associated with contraction at all. Verzár and Peter thought their smooth records obtained in a formaldehyde-treated muscle could be accounted for by the DeMeyer phenomenon; and they adduced experimental evidence that this might in part have been the case. However, the

deformation potential, amounting to much less than a millivolt, is far too small to account for the potential values found by Bishop and Kendall in the same experiment. Furthermore, a large-sized contracture potential occurs when the muscle is working as nearly isometrically as it is possible to attain. Bishop and Kendall consider the potential to be one associated with the contraction phase.

E. Fischer considered the currents produced by K, NaOH, HCl, and chloroform as injury currents. Riesser and Steinhausen, who found that the proximal or active end of a frog's gastrocnemius, treated with acetylcholine, became positive to the distal or inactive end, argued that this potential could be attributed to excitation of the active portion. But after reinvestigating the subject, Steinhausen came to the conclusion that the positive sign of the potential was a deception produced by the penniform structure of the muscle and that the essential change was really negative. He then reversed his previous opinion and considered the current as an injury current, although his records show a good parallelism, as to time, between the current and the contracture. Against such an interpretation of the acetylcholine potential Schaeffer and Licht have presented the following argument. When the mammalian tongue is denervated, acetylcholine injections do not produce a potential until sensitization has progressed to such a point that contraction results. Then the same potential may be produced repeatedly in successive experiments on the same muscle.

CONTRACTURES AS A RESPONSE TO STIMULATION. Following Bernstein (1890) most physiologists who have dealt with the contracture problem have considered the muscle as stimulated by the agents producing the contracture. We have already seen in support of Bernstein's view that three of the signs of activity, namely, heat, tension, and metabolic changes, follow the exposure to such agents; therefore, it is quite to be expected that there would also be an electrical sign. For this reason the interpretation of the experimentally observed potentials as adventitious or as evidence of injury cannot be passed without further comment.

The problem, briefly stated, is whether the contracture potential can be an electrical sign of muscular activity. Opinions to the contrary are undoubtedly based on the idea that the normal electromyogram is produced by a wave of excitation preceding contraction. Therefore, if the interpretation of the normal action potential can be shown to be incomplete, the opinions concerning the contracture potential must also be due for a revision.

In 1887 F. S. Lee, after an examination of frog muscle with the capillary electrometer, concluded that there is a close parallelism between the electrical and mechanical manifestations; and recently Einthoven (1924) has gone so far as to conclude that the muscle potential is entirely due to contraction. This conclusion is based on two experimental considerations. The first is inseparability of the electrical and mechanical responses, an opinion reached by Einthoven and Hugenholtz (1920) and by Arbeiter after a review of the literature and further experimentation. The second is the shortness of the latent period. Einthoven claims that in the newer determinations the latent period has been found to be sufficiently short to approximate the utilization period of the electrical response, and that therefore the supposition that there is a difference in the time of onset of the electrical change and contraction is no longer justified. An examination of the actual data, however, shows that there is a measurable delay of the contraction. Kleinknecht, using improved methods, found such a delay; and Fulton (1925), after correcting for every experimental lag for which there was justification, found a time difference between the beginning of the electrical and mechanical responses of 1.5σ. The observed latent period quoted by Einthoven is 1σ; so, as Fulton remarks, no one has yet actually shown that the two manifestations do actually begin with absolute simultaneity.

Recently Bishop and Gilson (1927) have studied the frog's sartorius, making every possible effort to arrive as closely as possible at the true monophasic form of the action potential. They find, like Lee, that the negativity lasts throughout the phase of shortening of the muscle, and in their interpretation adopt a view midway between the usual view and that of Einthoven; namely, that the potential is made up of two parts: an initial part, representing an excitation process corresponding to that in nerve, and a later part associated with the contraction process. A similar two part theory of the muscle potential has also been formulated by Schenck (1895).

When the problem of contracture potentials is now reëxamined with these experiments in mind it takes on some new aspects.

If the second part of the electromyogrm be associated with contraction, it is to be expected that it would appear in an exaggerated form associated with the prolonged contracted state in Tiegel's contracture. The subject has been but little studied. Bishop and Gilson,who were investigating normal twitches, found that the height of the portion of the muscle potential curve immediately following

the spike was greater when the lead was made close to the stimulating cathode. Evidently in this location the response was altered by the effect of the stimulation current. Therefore, it is not beyond the realm of probability that frankly supramaximal shocks might cause a still greater or more prolonged potential change associated with Tiegel's contracture. Beritoff states that the latter is not accompanied by an action current; but, as he gives no details, one is not sure that the lead was made so that it would record a localized response. Certainly, in analogy with the potential found in the formaldehyde contracture, a potential accompaniment of Tiegel's contracture is to be expected.

In an aldehyde-treated muscle, according to Bishop and Kendall, the second part of the muscle potential is continued throughout the contracture, the electromyogram following in its contour the various mechanical manifestations found in the varied modes of response. On account of this continuity and the general similarity of the contraction and contracture potentials, the authors infer a similar origin.

Considerable interest, therefore, centers around the second part of the electromyogram. But unfortunately the origin of this potential is only a matter of conjecture. Biedermann (pp. 322 and 331) in discussing Lee's results considers the potential simply as the expression of an excited state. On the other hand Bishop and Gilson speak of it as the sign of an electrochemical reaction underlying or accompanying contraction. Further elaboration of these opinions is wanting.

In line with Biedermann's view we may imagine that the muscle membranes remain permeable for some time after the conduction process, revealed by the spike, is completed; and this permeability according to the membrane hypothesis would have a potential sign. But as to why such should be the case we have no hint. In the veratrin contracture the permeability would last for a longer period and to a greater degree,—and here, as we have seen, there is a greater and more prolonged heat liberation than occurs in a twitch.

The view of Bishop and Gilson is less easily visualized, because the attention is focused on the phenomenon of contraction without any concrete suggestion as to the mechanism of the associated potential. When, however, we recall that there is no evidence that excitation and contraction may be separated, the views of Biedermann and of Bishop and Gilson are seen not to be mutually exclusive, provided we make the reservation, in keeping with the postulate of two processes made by the latter, that the state of excitation is not the same throughout the duration of a twitch as it is during conduction.

There are certain advantages for the purposes of discussion in speaking of the potential associated with contraction in terms of the membrane hypothesis, that is, as a potential due to the increased membrane permeability which is leading to contraction. Garten's observation on the action potential of veratrin-poisoned nerve then falls directly into line with the effect of the drug upon muscle. What Garten found was that, when such a nerve is stimulated with a single induction shock, the response is characterized by a great prolongation of the falling phase of the action potential. This can only mean a continuation of the response normally found in nerve as there is no possibility of complication by deformation, demarcation, or contraction potentials. We may therefore infer that muscle differs from nerve only in the fact that this prolonged excitation must be associated with contraction.

Allusion was made, in the foregoing discussion of the two-part theory of the electromyogram, to the possibility that excitation is not always the same thing. That such is the case was suggested by Jensen in his discussion of thermal contractures, with special reference to the differences between twitches and contractures in general. Jensen's theory is essentially one postulating two modes of approach to a single metabolism or mechanism. The first form of excitation is conceived in the spirit of the Nernst hypothesis as associated with the mobilization of electrolytes and attended by conduction. The second form is considered to be a local alteration in a labile complex without sufficient electrolyte accumulation to produce action currents large enough to stimulate contiguous regions. Accordingly, whether a twitch or a contracture is obtained, depends upon the immediate state of the muscle and the nature of the stimulation.

A theory basically analogous to Jensen's is used by A. V. Hill (1926) to explain the difference between the normal mode of energy release and the situation in the caffeine contracture. Making use of the materials afforded by the membrane hypothesis he has drawn a picture of diagrammatic clearness. In a twitch a wave of permeability sweeps along the muscle, permitting a transitory reaction of substances normally restrained (vide also Fulton, 1926, p. 235); while in the caffeine contracture there is a slight impairment of the membranes with a continuous energy mobilization. That these two conditions would be attended by a potential change is obvious. The wave of permeability would produce the spike of the normal electromyogram, and the slight impairment of the membranes in the caffeine

contracture would produce a continuous potential. In many ways the latter would be analogous to the second part of the aldehyde potential curve. The aldehyde electromyogram starts with the spike characteristic of the twitch preceding and initiating the contracture and ends with a potential persisting throughout the contracture phase. In a caffeine contracture developing spontaneously, it is to be expected that the spike would be absent as it is known to be in some other contractures.

In a recent contribution to the veratrin problem Kodera and v. Brücke have proposed a theory which also postulates two forms of irritability in muscle. They observed that when the time-strength curve of stimulation is determined in a mildly poisoned muscle (*rana temporaria*) two distinct curves appear, indicating two chronaxies. The first one is for the twitch response and has a value practically identical with that for normal muscle, e.g., 2.2σ. The second one, the chronaxie of the veratrin-effect is much larger and has a value in the region of 50σ. Initially a given strength of stimulus must last a longer time to elicit the prolongation of the twitch, than is necessary for the twitch itself; but as the poisoning is intensified the two curves approach each other, as one would expect, in view of the fact that at this stage any stimulus which is effective at all causes a typical veratrin response. In line with the opinion that the veratrin-effect is a tetanus, the authors think of this second form of excitation as a "tetanogenic" mechanism, and associated it with the closing tetanus in normal muscle; but with a very small alteration the theory could be made to fit the explanation of the veratrin-effect favored in this review. The normal analogy would then be with the constant-current contracture (compare Bremer's experiment with the constant current (p. 40)).

Now is the potential observed in a chemical contracture an action potential or an injury potential? As we have seen, there is considerable sentiment in favor of the latter view. E. Fischer argues that if the potential change be a sign of excitation then its time-course should correspond with the mechanical response; and from his observations he concludes that the dissociation of the two phenomena is too great for this to be the case. KCl produced a potential difference when a portion of a muscle was treated with a mixture containing one part of an isotonic solution and thirty-two parts of Ringer's solution, while a contracture was only produced with certainty when the solutions were mixed in equal parts. When a contracture appeared it reached its maximum long before the maximum of the

potential. The potassium potential started approximately simultaneously with the contracture, but a chloroform potential started as long as seconds to minutes before the mechanical change, the time depending upon the concentration of the chloroform. On the other hand N/100 HCl and N/100 NaOH produced a potential change only a considerable time after the start of the contracture.

Several points suggest themselves in connection with the above experiments. Potentials were observed when the KCl concentrations were too low to cause contracture and when the mechanical response in a chloroform treated muscle had not yet commenced. The concomitant phenomena were not observed, but it is most probable that during this period the rate of lactic acid production was increased and this would mean increased heat production. The absence of a mechanical change could then be accounted for by too slow a mobilization of energy, and there would thus not be a significant dissociation of the signs of activity.

A second reservation is necessary when we recall the warning given by Einthoven and Hugenholtz as to the sources of error in the comparison of the time-relations of two signs of activity, when they are recorded with instruments of different relative sensitivity. Galvanometers, if sensitive, are apt to record potentials when no contraction is visible, even when a light isotonic lever is used; and this fact might explain part of the interval found between the two manifestations in the case of chloroform, which was studied with a sensitive galvanometer. But there is the dissociation in the other direction in the case of N/100 HCl and N/100 NaOH. If this observation could survive all criticism, then we would have to admit that there is no connection between the contractile and electrical responses. *A priori* a contracture without a potential occasions some surprise in view of the fact that strong acids and alkalies are believed to injure the plasma membrane before they penetrate. Therefore, the experiment was repeated by the reviewer. E. Fischer used a string galvanometer with apparently a very tense string. In the repetition a more sensitive string was employed and it was then found that records of the contracture and the potential changes started approximately simultaneously. In another experiment in collaboration with Dr. G. H. Bishop, in which a very sensitive potentiometer was used, the first detectable potential change preceded the first detectable mechanical response. Therefore, the relative time of appearance of the two events is very dependent upon the technique; and it is possible for a lever to be relatively more sensitive than a galvanometer.

That the curve of shortening should differ from the potential curve is not surprising in view of the irreversible processes occurring in the muscles, and in view of the fact that the substances employed would produce marked potential changes in physical systems where there are phase boundaries or semipermeable membranes (e.g.,Beutner, Michaelis), systems which are widely different from living muscle. Such potentials could easily complicate and obscure any potential connected with activity.

The import of the foregoing discussion is that the experimental data do not preclude the possibility of the existence of a potential connected with activity. Whether the potential be called an action potential or an injury potential is not of great moment. From the standpoint of the physical chemistry of muscle there is no clear differentiation. We may quote in this connection a statement made thirty-three years ago by Biedermann (p. 331). "There is as little fundamental difference between an action-current and a demarcation-current as between stimulated and dying muscle-substance. From this standpoint it is immaterial whether or not the 'potassium-current' of muscle is called an action-current."

Note on the separability of conduction and contraction. In the foregoing discussion conduction and contraction have been spoken of as occurring in sequence. But it must not, therefore, be assumed that they are independent and separable at will. We have already mentioned Einthoven and Hugenholtz' objections which negate experiments purporting to separate the action-current from contraction. The same type of objection holds for experiments supporting the separation of conduction and contraction. In such experiments one end of a straight-fibered muscle is poisoned, and on stimulation is found to cause contraction in the intact end, without itself contracting. The difficulty arises from the fact that a conducted impulse, accompanied by a contraction too weak for the recording apparatus, on emerging into normal tissue becomes full-sized again and easy to detect. When the evidence is weighed with this point in mind, it seems to show that conduction cannot take place without contraction. (The literature on this subject has recently been reviewed by Fulton (1926, p. 288) with this conclusion.) *On the other hand every contracture is an instance of contraction without conduction.*

There are also other conditions in which separation of the signs of activity is evident. E. Fischer's experiments showing a potential change in muscles treated with subthreshold KCl solutions—whether

or not this potential be considered as associated with activity—demand the admission that a potential change is possible without contraction. Furthermore, the inseparability of heat and tension, found by Gasser and Hartree to hold when a muscle poisoned by alcohol or hypotonic solutions is stimulated electrically—even when narcosis is so deep that contractures must be produced by the strong stimuli—can apply only to the response of a muscle to stimulation. An anaerobic muscle accumulates lactic acid without tension-production, and the lactic acid must determine an evolution of heat. The absence of tension is explained by Meyerhof and Matsuoka as due to a too slow velocity of lactic acid evolution. It is possible that in mild potassium-poisoning a similar situation may obtain.

In brief, attempts to separate the signs of activity have resulted in demonstrating that if a muscle be excitable there must be sufficient integrity in its membranes (or their theoretical equivalent) to hold the forces of the muscle in leash. Therefore on excitation energy will be mobilized with all the usual signs, provided it is mobilized at a sufficient velocity. It is not necessary that conduction take place. But if it does take place it will the more surely be followed by tension production.

Objections to the view that the contractures are a response to stimulation have been raised by Bethe and his co-workers. The most extensive statement of their arguments is to be found in the paper by Bethe, Fraenkel and Wilmers; but other aspects of the problem are presented in the papers by Schwencker, by Wilmers and by Bethe (1923). They cite three principal objections. *1.* The contractures are not conducted. *2.* They can be produced in muscles rendered non-irritable to electrical stimulation by narcosis or otherwise. *3.* They can be produced by muscles in a state of extreme fatigue. The only effects of chemical substances which they admit as stimulations are the fibrillary twitchings which possess *all* the characteristics of a stimulated muscle. The other effects are considered to be directly upon the contractile elements in the muscle.

We may pass over the exclusion of the contractures from the category of stimulation, since it is largely a question of definition which is involved. We have seen that, aside from the acid-contracture, contractures are characterized by shortening or tension production accompanied by heat development, utilization of the muscle's special metabolism, and most probably by an action potential. To argue further what such a state of the muscle should be called adds nothing to our knowledge of the subject.

In separating off from the chemical contractures a special group of stimulated contractures (produced by acetylcholine, nicotine, etc.) Riesser, conceiving that stimulation of muscle must have all the properties of a twitch response, met the conduction difficulty by attempting to show that conduction is also not a necessary property of a twitch. With Richter he was successful in producing in one end of the frog's sartorius twitches which were not conducted throughout the length of the muscle This observation, if it mean failure of conduction in fibers running the length of the muscle, at once strikes one as being in contravention of the all-or-nothing law, and it would have such a far-reaching significance in the physiology of muscle that one wonders whether the result may not have been due to short fibers, a few of which are stated by Adrian (1925) to exist in the frog's sartorius. It will be seen later that acetylcholine acts on anesthetized and fatigued muscles like many other substances.

Contractures occurring in narcotized muscle. As the experiment is performed by the various observers, narcosis means the failure to respond to an electrical stimulation which had been initially maximal. In this state we have seen that the muscles can still be made to contract by alkalies, by chloroform, by narcotics in high concentrations, by heat and by mechanical stimuli. The contracture can also be produced by acetylcholine (85).

Bernstein, who was familiar with the chloroform contracture of etherized muscle, met the excitation problem by pointing out that there is no reason to believe that the irritability of a muscle should disappear simultaneously to all forms of stimulation; and later Tissot observed that a depressed muscle loses its power of response to electrical, thermal and chemical stimuli in the order mentioned. This order cannot stand, however, without some explanation, as it depends both on the strength and the duration of the electrical stimulus under consideration. For instance, Saito readily produced responses with constant currents in muscles sufficiently narcotized with amylene hydrate or urethane to eliminate the initial twitch. The absence of the twitch, characteristic of the reaction of a normal muscle to such a stimulus, established the contracture-like nature of the response; and, as was to be expected, the contracture was local. When recorded with a double myograph the shortening in a muscle, stimulated end to end, occurred on the cathodal side only, if currents of 0.1 m.a. or less were used. Currents of 0.2 m.a. and stronger produced some shortening of the anodal half, also; but this must have been due to an extension of the field of stimulation.

Recently H. T. Graham has made a number of observations of the behavior of narcotized muscle stimulated by induction shocks. She found that when either the narcotic gases or ether were employed, "complete" narcosis was never attained. No matter how far the narcosis was pushed, a sufficiently strong shock always evoked a response. In fact, the depth of narcosis and the strength of stimulation could be increased until the narcotic itself began to produce contracture and the shock began to leave traces of irreversible injury. The response of the narcotized muscle differed from a normal contraction in that it was smaller and more prolonged. A number of experiments were performed with a double myograph on muscles stimulated with induction shocks applied through electrodes, both of which were at the same end of the muscle. In the normal muscle, any stimulus producing a response at the cathode also caused a contraction throughout the muscle; but as narcosis developed the response always failed first in the unstimulated end. The curves describing the thresholds of response for the two ends began to diverge. This divergence was slight at first but as the threshold for the stimulated end progressively rose, the divergence became relatively greater and at the depth of narcosis, at which the threshold for the proximal end began to rise rapidly, it was practically impossible to obtain a response in the distal end at all.

The experiments just described reveal the fact that treatment of a muscle with a narcotic produces a progressive impairment of conduction in the muscle as a whole. While all the steps in this impairment have not been analyzed, and although the course of the impairment in any one fiber may be different from that manifested by the entire muscle, it is clear that the end condition is one in which the response which is obtained is entirely local. It therefore has the general properties of a contracture. Thus a better statement of the relation of the contractures to narcosis would be that, following narcosis of the contraction associated with conduction of excitation, contractures may still be evoked by electrical, chemical, mechanical and thermal agents.

Contractures in fatigued muscle. C. R. Marshall showed that a frog muscle stimulated tetanically to fatigue contracted again in tetramethylammoniumchloride, and this result has been duplicated by applying acetylcholine to a muscle so fatigued that a five second gap in the stimulation permitted neither relaxation nor visible recovery (85). In a similar experiment Bethe (1923) found that chloroform

restored the tension to the original tetanus value. A complete restoration of tension was also observed by Gasser and Dale in fatigued denervated mammalian muscle responding to injections of members of the nicotine group. Here the restoration was completely reversible. It was also transitory. The tension soon fell to a level below what would have obtained had the stimulation been continued without the drug injection; but some recovery from this low level slowly occurred in spite of the tetanic stimulation which was maintained throughout the experiment.

The foregoing contracture experiments lead one to believe that the fatigue of a tetanus is a problem of excitation. The low tensions of fatigue would easily be explained by the assumption that the excitation mechanism is so altered that the amount of energy liberated at each wave of activity is too small to permit anything larger. The increased rate of fatigue with higher frequencies of stimulation would fit with the hypothesis because of the resulting smaller size of each excitation wave; and for the opposite reason the greater exhaustion of the muscle energy stores which can be produced by slow stimulation (Hartree and A. V. Hill, 1924; Meyerhof, 1925) would be explained. Contractures of fatigued muscle would be accounted for by the substitution of an adequate for an inadequate stimulus. There is still a large energy reserve in ordinary fatigue (Fletcher and Hopkins). It only needs an appropriate stimulus to be released.

THE IRRITABILITY OF MUSCLES IN CONTRACTURE. When a muscle is stimulated electrically, there is a period after the response during which a second response is impossible or subnormal. This is known as the refractory period. Similarly, when a muscle is in contracture, the activity induced by electrical stimuli is abnormal. Such a muscle is also refractory, and the properties of this refractory state throw considerable light on the nature of the contracture, itself.

Schiff (1857) found—and his finding has been confirmed by P. Hoffmann (1913)—that a wave of excitation cannot pass over a mechanically produced (ideo-muscular) contracture. Querido found the absolutely refractory phase considerably prolonged in the veratrin-twitch. Second responses occurred during the contracture, but were smaller than they would have been in a normal muscle at the same period. Schwenker observed that the effect of electrical stimulation was greatly reduced or absent at the height of the contractures from acids, alkalies, alcohols, bile, etc., and a similar effect was described by Langley (1908) for nicotine.

The greatest interest is attached to the more perfectly reversible contractures of the acetylcholine group. Interesting experiments on a member of this group have been described by C. R. Marshall. In a contracture produced in a frog's sartorius with tetramethylammoniumchloride, twitches stimulated by break shocks decreased greatly at the height of the drug action; but as relaxation occurred they returned to normal size in spite of the continued presence of the substance. A very similar effect has been found by Gasser and Dale in the rectus abdominis treated with acetylcholine. In frog and fowl muscle, where the tensions produced by acetylcholine are low as compared with those of a tetanus, the decrease in the height of short tetani, excited during a contracture, is small. In normal mammalian muscle, which is insensitive to acetylcholine, there is no decrease at all. But, if fowl or mammalian muscle be sensitized by denervation, the tension induced by the drug is large, and the responses to electrical stimulation are annihilated while the effect of the drug is maximum. The refractoriness cannot, however, be explained by the amount the muscle has contracted. A similar contraction produced by electrical stimulation does not depress for so long a time the effect of a subsequent electrical stimulation; and acetylcholine, so slowly perfused through a sensitized mammalian muscle that it produces only a minimum of tension, still greatly reduces electrical excitability. Also, the general group of directly-acting contracture-producing substances, which usually induces only low tensions, still renders the responding muscle refractory. In the production of refractoriness the *mode* of stimulation must be a very important factor, as well as the degree.

Denervated mammalian muscle is a good object for the study of the refractoriness in acetylcholine contractures, because of the high tensions produced, the good reversibility, and the known metabolism. An intra-arterial injection produces a transient contracture but the relaxation is not due to the destruction of the drug, since the muscle is at first perfectly refractory to acetylcholine itself. When the muscle passes from the refractory state, the irritability to acetylcholine is regained as is also that to electrical stimulation, but the return of the latter is strikingly slower. A condition is produced wherein the strongest electrical excitation will produce but a small effect, while an injection of acetylcholine will evoke a much larger, though still somewhat subnormal, response. The drug can be thought of as having altered the irritable surfaces so that a chemical stimulus is much more adequate than an electrical one.

The recovery of electrical excitability by a muscle still immersed in acetylcholine indicates that the drug action ceases when physicochemical equilibrium is attained. Refractoriness of the muscle and the stimulating power of the drug disappear together (86) suggesting that they are alike attributable to a change in state of some muscle structure; for instance, an alteration of some membrane during the period of penetration of the drug, or of some surface during the period of adsorption.

CONTRACTURES AND THE ALL-OR-NOTHING LAW. Gradations of tension in a twitch are due to the number of elements active, but in a contracture this is apparently not the case. We have seen how the Tiegel contracture varies with the strength of the stimulus. Analogously in the chemical contractures the degree of response is dependent on the concentration of the stimulating substance (vide Langley, 1913, for data on nicotine; Riesser and Richter for K, CNS and acetylcholine). The variations cannot be due to involvement of a portion of the fibers only, because the low grade activity of the muscle, induced by weak solutions, is still associated with a high degree of refractoriness (86). Furthermore, the low maximal reversible tensions produced by many chemical agents are not raised to anywhere near a tetanus value by further electrical stimulation. All, or at least a large portion of the muscle fibers must be involved, but not with maximal tension production.

In other words, the contractures do not obey the all-or-nothing law.

This brings up the question of why a muscle ever follows the all-or-nothing law. Adrian (1922) reasoned that "The stimulus and the resulting contraction are at opposite ends of a complex chain of events in the muscle fiber, and an all-or-nothing relation between the initial and final event might be due to an all-or-nothing relation occurring at any point in the chain." He then obtained evidence as to the location of this point by studying the sizes of the action currents in the twitches of a single fiber. They were found not to vary with the strength of the stimuli. He, therefore, suggested that the all-or-nothing relation occurs very early in the chain of processes.

Adrian's suggestion may be visualized by restating it in terms of the hypothesis of membrane permeability, previously quoted from A. V. Hill. The constant value of the action potential means constant permeability of the membranes during the conducted wave and therefore a release of energy in constant amounts. In the contractures, where the conducted wave is absent, it is not surprising

that responses of variable magnitude may be evoked in single muscle units.

The all-or-nothing law in muscle is based on experiments showing step-like increments of response to continuously increasing stimuli applied directly or indirectly, and a maximum effect independent of the strength of stimulus when the stimulus is applied through the nerve (Lucas, Pratt and Eisenberger). The muscle response is thus augmented by new units. But the range of strengths of stimulation, to which any one fiber is subjected, is not large and there would be little opportunity for the development of the Tiegel contracture. If it did occur the height of the twitch, which is the thing measured, would not be altered; on the other hand the tension-time area would increase. This change would affect the summation of twitches, and it may be for this reason that it is so difficult to obtain a definite maximal tension in a tetanus.

DUALITY. The dual theory of muscle activity, which distinguishes fibrillary from sarcoplasmic contraction, was devised by Bottazzi to explain the veratrin-twitch. But its usefulness in that particular case has been eliminated by the demonstration that the various aspects of the veratrin-effect may be explained without involving the sarcoplasm. The theory still persists, however. On account of its very simplicity it affords convenient explanations, of an illusory satisfactoriness, for various phenomena not well understood. These have recently been ably criticized by Hansen, P. Hoffmann and Weiszäcker.

As far as the contractures are concerned, the remaining stronghold for the dual theory is in the response evoked by acetylcholine in frog muscle. It is true that the energy source of the latter has not been found; but this does not constitute positive evidence for a dual mechanism.

DO CONTRACTURES OCCUR IN VIVO? Ideomuscular and Tiegel contractures are clean-cut cases of contractures occurring in the body, so also are the responses of denervated mammalian muscle to chemical stimulation. It is not with respect to these examples that the question is raised, but rather with regard to the pathological sustained contractions or shortenings which occur clinically or may be produced experimentally. These are generally designated as contractures; but if critically examined usually fail to qualify under the restricted definition, which is the guide for this review.

Sustained contractions have been extensively studied in connection

with the physiology of tonus, where the question at issue is whether such contractions can occur without involving the *known* tonus mechanism. The latter as recently defined by Cobb in these REVIEWS consists of a beautifully graded series of proprioceptive reflexes moulding individual muscles. It must therefore manifest a rhythmic electromyogram. Accordingly the action potential has been employed to differentiate tetanic tonus from other forms of a hypothetical nature, not so innervated.

The tonus problem as such is entirely outside the scope of this review, but the electromyographic data derived in its behalf are of the greatest usefulness for the differentiation of tetani from contractures. The details as to individual cases may be found in recent reviews by Hansen, P. Hoffmann and Weiszäcker and by Fulton (1926, 413 ff.). When summarized they indicate that the tendency of recent investigations has been to show more and more of the pathological sustained contractions to be of tetanic origin. Contractures would, therefore, be excluded. But the objection has been raised that, even if one grant the unimpeachable validity of the data under consideration, it is still possible for a tetanic contraction to be engrafted upon a shortening of some other origin. Such a possibility, it must be admitted, is not eliminated by the electromyogram alone. A decision must rest on other information. However, when a sustained contraction has been demonstrated to be a tetanus, a sufficient explanation has been found, and there is no reason for classifying it otherwise until experimental evidence indicates the existence of some other concomitant element.

Muscle shortenings distinctly not of a tetanic nature are found in the group recently designated by Ranson (1928) as "myostatic contractures." After a muscle has been shortened for some time as the result of division of its tendon (Ranson and Dixon), fixation in a plaster cast (Fröhlich and Meyer, 1916; Spiegel and Shibuya), or poisoning with tetanus toxin (Fröhlich and Meyer, 1919; Ranson and Morris) a condition supervenes in which relaxation to the normal length does not occur in anesthesia or after cutting the nerve. The muscle is still irritable; it contracts and relaxes when stimulated, but the relaxation stops at the new length. This length, moreover, must be determined by the muscle fibers themselves as there is no evidence of fibrosis or of a pathological state of the joints.

A necessary condition for the production of these "myostatic contractures" is the integrity of the innervation. Section of the

motor nerve prevents their appearance and they are greatly reduced when the dorsal roots are cut. On the other hand simple fixation at a decreased length is insufficient. Obviously the latter must be attended by a prolonged innervation, and that such is the case has been demonstrated electromyographically in tetanus by Hansen, P. Hoffmann and Weiszäcker, and in the retraction following division of a tendon, by Rehn.

But when the shortening has progressed to the second stage, that is, when it persists after denervation, a rhythmic electromyogram would of course be absent. It would therefore follow that one is not always to be expected when the innervation is intact. Such, indeed, might be the explanation of the negative findings of Fröhlich and Meyer, and Liljestrand and Magnus in muscles poisoned with tetanus toxin; but it not clear that such is the case, as it is not certain that the muscles examined would have failed to relax had their nerves been cut. It does hold in the most recent experiments of Keller. The latter, using amplification in connection with the string galvanometer, always found action potentials in tetanus-poisoned muscles, provided they were contracted and had not passed into the permanent secondary stage. Thus permanent shortening should always be considered in explanation, whenever smooth electromyograms, beyond technical criticism, occur clinically in cases of spasticity.

Now, what is the nature of this condition produced by long innervation? Fröhlich and Meyer (1920) considered that the muscle had changed the length of its resting position and that the shortening was not the expression of a contraction using energy, developing heat and easily fatiguing. This opinion was supported by v. Brücke; and recently it has received experimental confirmation in some experiments by Davenport, Davenport and Ranson in which it was shown that no increase in lactic acid occurred as compared with a control muscle. Therefore the "myostatic contractures," legitimately so styled according to current usage, do not fulfill the conditions we have set for the limited application of the term "contracture." As resting states and not involving activity of any part of the muscle's contractile mechanism they differ too greatly from the condition produced by caffeine, for instance, to be designated by the same term.

Dittler and Freudenberg have described a condition which might be considered a contracture. They found that the spasm produced by over-breathing, as in the experiments of Grant and Goldman and

of Collip and Backus, persisted after the motor nerve had been blocked by novocaine, while the electromyogram, which previously had been rhythmic, became smooth. This meant a local action on the muscle, an action which the authors ascribed to the alkalosis or to deionization of the calcium. However, Freudenberg and Läwen found that the spasms produced in dogs disappeared when the nerves were blocked; and Flick and Hansen, on repeating Dittler and Freudenberg's experiment on man, found that, when the electromyogram became smooth, the contraction disappeared. Therefore the existence of a contracture element in tetany must be considered as extremely doubtful.

AFTER-WORD. In the foregoing review a large amount of controversial material has been considered, and data have been adduced furnishing pathways to many alternative opinions. On the basis of these data an argument has been presented, which may be compared to an itinerary selected on a route with many divisions. At each division the alternative has been followed which seemed to have the most in its favor; but, for the aid of the reader who may consider some other alternative as the most favored, guides to contrary opinions have been erected at each division. If these be not chosen and the reader at each juncture take the same path as the reviewer, he will arrive at the conclusion that the known contractile mechanism in muscle can be set into states of activity differing in their properties from those resulting from the normal motor innervation. These states of activity constitute the major portion of the conditions which can be designated as contractures under the restrictions placed upon the term in the introduction. They differ from a tetanus chiefly in the absence of conduction and in properties believed to be secondary to this deficiency.

While the contractures are abnormal responses of muscle, an understanding of their properties has two merits. It augments the understanding of the closely allied normal twitch and prevents the confusion of qualities, properly belonging to a contracture, with those possessed by a twitch. It also reveals the fact that a muscle set into activity by pharmacodynamic agents may follow laws different from those believed to govern a muscle stimulated through its nerve.

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THE ENZYMATIC SYNTHESIS OF PROTEIN

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The study of enzymatic synthesis is of especial interest in the case of the proteins because of the great complexity and size of protein molecules as compared with other biologically important chemical groups such as carbohydrates and fats, and because of the intrinsic importance of protein synthesis in the building up and maintenance of the structure of the organism.

Biochemistry in the past has found its main occupation in analytical studies, i.e., in the isolation and correlation of the products of decomposition in the organism. With the fats and carbohydrates this has been instructive because their main function is the liberation of energy in the formation of degradation products, and it has been facilitated because means have been readily available for simulating *in vitro*, the decompositions occurring *in vivo*.

Analytical studies applied to the protein molecules have succeeded to a large extent in elucidating the essentials of its structure, and an enormous volume of information has been accumulated regarding the decompositions effected by hydrolysing enzymes and their mode of action. Much less progress, however, has been made with the biochemistry of the proteins than with that of the fats and carbohydrates.

The main function of the proteins is associated with their participation as such in the structure of the organism, and elucidation of the synthetic processes is consequently essential for an understanding of the mechanism by which this participation is secured. The study of *in vivo* synthetic processes, however, labours under the difficulty that biological syntheses can, only imperfectly, be simulated *in vitro*.

In the anlaytical studies attention has been directed to the agencies and substances involved, rather than to the general laws defining the course of reactions. In the study of enzymatic synthesis, on the other hand, the development of theory has been in advance of, and has guided experimentation from the first. The essence of the theory is the applicability of the Law of Mass Action to enzyme reactions, and their

reversibility consequent upon the assumed catalytic nature of the reaction.

The achievement of the enzymatic synthesis of disaccharides (1) and of fats (2) was due to the stimulus given by this theory. The first observation of the enzymatic synthesis of protein was made by Danilewski (3) independently of theory, but the theory was very soon called upon to support the contentions that the reaction under observation was in fact an enzymatic synthesis, and that under the conditions of Danilewski's experiment, synthesis was to be expected.

Theoretical aspects of polymolecular synthesis. In applying the mass law to the hydrolysis of protein the equation is often written Protein + $H_2O \rightleftharpoons$ Products. From this equation it has been commonly assumed that sufficient diminution of the concentration of water would cause the reaction to proceed from right to left. The fact is usually overlooked that the molecular concentration of the water is enormous when compared with the concentration of the other substances and, therefore, even with tenfold concentration of the solution the ratio of the molecular concentrations is so little altered that reversion, by this mechanism alone, would be practically impossible *in vitro*.

The opinion is occasionally expressed that protein synthesis must be especially difficult because the hydrolysis of proteins goes to practical completion. The argument is somewhat as follows: The equilibrium point is apparently at 100 per cent decomposition and the catalyst does not influence the point of equilibrium. The reversion of the reaction is consequently difficult of accomplishment.

To account for synthesis in the tissues, there has been postulated the influence of surfaces for the reduction of the concentration of water to a point so low that molecularly it would be of the same order of magnitude as the concentration of the products and the protein. An example of this reasoning is found in Bayliss' *Principles of General Physiology* (4). In an alcohol-water-acid-ester system Bayliss deduces the equilibrium constant as $\frac{(\text{alcohol})\ (\text{acid})}{(\text{ester})\ (\text{water})} = \frac{K_1}{K_2} = K$. He then states:

> Put in this form, we see that if we increase one component, the result must be to decrease its fellow, since the value of the fraction *must remain unaltered.* Suppose we increase water, the value of this fraction can only be kept constant either by increasing (alcohol) (acid) or by decreasing (ester). In point of fact, of course, the two are identical, since one cannot take place without the other. The results of excess of water should be, therefore, to increase the hydrolytic reaction of the system, as found by experiment.

The conclusion to be drawn from this fact is that, in order to obtain much indication of the synthetic aspect of enzyme action, the concentration of water must be diminished as far as possible.

In the living cells, where synthetic processes readily take place, it seems that there must be some very effective means of doing this, perhaps by surface condensation or imbibition on the part of the colloids. But as yet we have no clear idea of the mechanism.

In this example the molecular concentration of water and of the other reacting components is of the same order of magnitude. In the protein systems usually encountered *in vitro*, however, if no special mechanisms are postulated, the molecular concentration of water must be enormously greater than that of the reacting components. If the essential conditions for synthesis were those demanded by Bayliss, reversion would, *in vitro*, be impossible of attainment, because the enormous value of the molecular concentration of water would always drive the reaction from left to right. To account for the occurrence of synthesis in the tissues it would then be obligatory to postulate, as Bayliss did, a special mechanism capable of effecting enormous diminution of the concentration of water, with a resulting increase in concentration of the substances on the right-hand side of the equation.

In the hydrolysis of protein, we have the formation of a number of components from one molecule of protein. This fact places the hydrolysis and synthesis of protein, and to a lesser degree of fats, in a special category. Moore (5) presented a theoretical formulation which in condensed form is as follows. In the simple reaction $A \rightleftharpoons B$ the energy and volume changes are represented by:

$$C = H + V \tag{1}$$

where C is the chemical energy set free in the passage of 1 gram mol. of A into B, V represents the change in volume energy, and H is the heat energy for the transformation at pressures defined by the V term. The disappearance of A causes a diminution of volume energy given by the expression $RT \log \frac{P_A}{P_0}$, and the appearance of B causes an increase of $RT \log \frac{P_B}{P_0}$, where P_A and P_B are terms proportional to the osmotic pressure of A and B respectively and P_O is a term proportional to the osmotic pressure at zero concentrations of A and of B. The net change in

volume energy is $RT\left(\log \frac{P_B}{P_0} - \log \frac{P_A}{P_0}\right)$, or $RT \log \frac{P_B}{P_A} = V$. Substituting in equation (1)

$$H = C - RT \log \frac{P_B}{P_A} \tag{2}$$

Equilibrium is attained when $H = 0$, or when $RT \log \frac{P_B}{P_A} = C$. At equilibrium the reaction is defined by this equation, which can be restated as $\frac{P_B}{P_A} = e^{\frac{C}{RT}}$ or

$$P_B = KP_A \tag{3}$$

Where one molecule decomposes into two, as in the hydrolysis of a disaccharide, the volume energy lost is, as before, $RT \log \frac{P_A}{P_0}$; but the volume energy gained is $2RT \log \frac{P_B}{P_0}$. At equilibrium this reaction is defined by the equation

$$RT \log \frac{P_B^2}{P_A \cdot P_0} = C,$$

or

$$\frac{P_B^2}{P_A \cdot P_0} = e^{\frac{C}{RT}}.$$

Therefore

$$P^2_B = KP_A. \tag{4}$$

No term is inserted for the water which may take part in the reaction because its molar concentration remains practically constant.

In equation (3) the ratio of the equilibrium concentrations of A and B is a constant, and is therefore fixed and independent of their absolute concentrations. On the other hand where one molecule decomposes into more than one, the equilibrium ratio as shown in equation (4), is not fixed and independent of the absolute concentrations. It varies in such a way that in dilute solutions the equilibrium is at a point where the substance undergoing decomposition, a disaccharide for instance, is

nearly completely dissociated into its constitutent hexoses. In more concentrated solutions the equilibrium ratio moves more and more to the side of conversion of the hexoses into the disaccharide. For instance, if, in the equilibrium equation (4) the concentration of the disaccharide is doubled, P_A becomes $2P_A$ and P_B becomes $\sqrt{2} \cdot P_B$ for P^2_B becomes $2P^2_B$, P_B then becomes $\sqrt{2P^2_B}$ or $\sqrt{2} \cdot P_B$ so that P_B here increases less than P_A. In other words the degree of hydrolysis is less. Conversely, on dilution P_B decreases in less ratio than P_A, or the degree of hydrolysis increases. In the case of the hydrolysis of a neutral fat the equilibrium equation becomes

$$P^4_B = KP_A,$$

since one molecule decomposes into four. Here the effect of concentration is more marked. In concentrated solutions the tendency to form fat is greater. In dilute solutions the tendency to form fatty acids and glycerine is greater.

The general equation is

$$P^n_B = KP_A \tag{5}$$

where n is a whole number greater than unity or the number of molecules of B into which one molecule of A decomposes. The consequences of equation (5), paraphrasing Moore, can be stated as follows: In those reactions in which the number of molecules in solution increases, the position of the equilibrium point depends upon the absolute concentration of the solution. Such reactions tend to become reversible or incomplete in concentrated, and irreversible (or complete) in dilute solutions. Proteins belong to the class of compounds where the value of n in equation (5) is high. The effects of concentration on the one hand, and of dilution on the other, apply to proteins with especial force.

A similar conclusion can be arrived at by applying the Law of Mass Action, as Bayliss did, if the conversion of a single molecule into many be not overlooked. The equation for the hydrolysis of protein may be written

$$\text{Protein} + H_2O \rightleftharpoons Pd_1OH + H \cdot Pd_2 + Pd_3OH + H \cdot Pd_4 \ldots\ldots$$
$$Pd_{n-1}OH + H \cdot Pd_n. \tag{6}$$

where n is the number of molecules into which one molecule of protein is split, and Pd is the symbol for one of these products. The equilibrium

equation becomes, if the molecular concentration of water be considered constant

$$\frac{(Pd_1 \cdot OH \times H \cdot Pd_2 \ldots\ldots\ldots \times Pd_{n-1} \cdot OH \times H \cdot Pd_n)}{(\text{Protein})} = K \quad (7)$$

or

$$\frac{(Pd)^n}{(\text{Protein})} = K \quad (8)$$

If the initial concentration be denoted by C_1, the degree of hydrolysis by h, equation (8) may be written

$$\frac{(h \cdot C)^n}{(1-h)\,C} = K \quad (9)$$

Transforming it

$$\frac{h^n\, C^{n-1}}{(1-h)} = K \quad (10)$$

or

$$C^{n-1} = \frac{K\,(1-h)}{h^n} \quad (11)$$

The consequences of equation (11) are illustrated in table 1 where hypothetical values are taken for h and n at a molar concentration of $C = 1$. The equation is solved for K and h is then calculated for other values of C.

It is obvious that h varies inversely as the initial concentration C. In terms of protein hydrolysis and synthesis, as the initial concentration of protein (or the concentration of products of hydrolysis) increases, the possibility of synthesis increases. If the initial concentration of protein is high, the degree of hydrolysis is small, and at equilibrium relatively little protein is hydrolyzed. If there is no protein at the outset of the reaction as is the case when a solution of the products of digestion is concentrated, then, in the presence of enzyme, sufficient protein is synthesized to provide the amount required to satisfy the equilibrium equation for the degree of hydrolysis existing at that concentration of substances in solution. As C increases in arithmetical progression, as it does when a protein digest is concentrated, h decreases in geometrical progession, and hence the tendency for synthesis, which is a function

of the reciprocal of h, increases in geometrical progression. Decreasing C in arithmetical progression the converse holds true. Therefore, where n is high, in concentrated solutions the tendency for synthesis is great and in dilute solutions the tendency for hydrolysis is great. The difficulties encountered in accounting on physicochemical grounds for synthesis *in vitro*, or in the tissues, thus vanish.

In addition to concentration, the other factors which affect the position of the point of equilibrium and determine whether a reaction shall be practically reversible or not, are the value of C, the chemical energy involved in the change from the state on the left hand of the equation to that on the right, and the absolute temperature of the reaction. The smaller the chemical energy involved in the change, the more does the reaction become practically reversible. The value of the chemical energy is not measurable experimentally. The heats of reaction as usually measured do not give either C or H, but instead the heat of reaction for the change of a gram mol. of substance at definite values of P_A and P_B. This figure is the only experimental datum available, but it varies in the same manner as C; a small heat of reaction indicating a small value for C, and a large heat of reaction, a high value. The heat of reaction for the hydrolysis of proteins (6) (7) is practically zero. Protein hydrolysates present in this respect, therefore, almost ideal examples of reversible systems. The effect of a change in temperature is opposite to that of a change in chemical energy. A rise in temperature should theoretically have the same effect as a drop in chemical energy, i.e., tend to increase the reversibility of the reaction. This was pointed out by Moore and is an obvious consequence of equation (5). In this equation, $P_B^n = K P_A$, K is a symbol for the expression $P_O e^{\frac{C}{RT}}$, P_O and e are constants, R is the gas constant, C the chemical energy involved in

TABLE 1

The effect of concentration on the degree of hydrolysis

INITIAL CONCENTRATION OF A C	NUMBER OF MOLECULES OF B ARISING FROM A n	AFFINITY CONSTANT K	DEGREE OF HYDROLYSIS h
1	10	9.91	0.99
2	10	9.91	<0.7, >0.6
4	10	9.91	<0.4, >0.3
8	10	9.91	<0.2, <0.1
16	10	9.91	<0.1

the breakdown of 1 gram molecule of A into n molecules of B, and T is the absolute temperature. It is obvious that an increase in T should have the reverse effect of an increase in C, i.e., should tend to increase the relative values of P_A, and to diminish P_B. Where C is very small, as it is in the case of protein hydrolysis, the effect of temperature on the position of equilibrium should be marked. A striking confirmation of these theoretical conclusions was obtained by Roberston (8) and by Borsook and Wasteneys (9), who found, in solutions where hydrolysis had ensued at a lower temperature, that synthesis could be induced by increasing the temperature.

The evidence of synthesis. A condition in which a synthesis of protein-like material occurs, was first arranged in 1886 by Danilewski (3) who observed the formation of a precipitate when stomach extract was added to a concentrated solution of the products of peptic hydrolysis. He believed that the precipitation was effected through the agency of an enzyme, because it did not occur if the stomach extract had been previously heated to 100°C. This result was confirmed in 1895 by Okunew (3). Both Danilewski and Okunew concluded that the reaction involved synthesis of the products of protein decomposition into a molecule whose complexity approached that of native protein. In 1901 Sawjalow (3) investigated the phenomenon more extensively and gave to the precipitate the name of plastein.

Sawjalow, using Witte's peptone in a concentration of 27 per cent, obtained as a precipitate nearly 14 per cent of the total dry weight of peptone. This formation of a precipitate in a concentrated peptic digest through the agency of pepsin was studied subsequently by Lawrow and Salaskin (10), by Bayer (11), by Robertson (8) (12), by Henriques and Gjaldbäk (13) (14) and by Wasteneys and Borsook (9) (15) (16) (17).

Kurajeff in 1901 (18) observed that papayotin, an enzyme of vegetable origin which resembles pepsin in its rennet-like action, also caused a precipitation in concentrated peptic digests. He found that, in contradistinction to the behaviour of pepsin, the most copious precipitates were obtained in alkaline reactions. Kurajeff claimed that the precipitate so obtained resembled that produced by pepsin in its property of forming a jelly when heated in a faintly alkaline solution. Similarly Gerber (19) found that extracts of vegetable tissues which coagulate milk, form precipitates when added to concentrated solutions of Witte's peptone.

Delezenne and Mouton (20) in 1907 found that activated pancreatic juice caused the formation of a precipitate when added to a concentrated

peptic digest. Subsequently hydrolysis ensued, the plastein precipitate disappeared, and crystals of leucine and tryosine were formed in the solution. Similar synthesis followed by hydrolysis was observed by Henriques and Gjaldbäk (14) and by Wasteneys and Borsook (21) when commercial trypsin was added to a concentrated peptic digest. Henriques and Gjaldbäk followed the rate of plastein formation and the accompanying change in the formol titration value. They found that the amount of plastein precipitated attained a maximal value in about three days. The rise in the formol titration value, indicating hydrolysis, began at about this time. Bayliss (22) observed that a concentrated solution of products of tryptic digestion of caseinogen shows a diminution of electrical conductivity under the action of trypsin. Similarly Marston (23) observed the formation of a precipitate and the reduction of the free amino nitrogen in the protein free filtrates (after addition of trichloracetic acid to 2 per cent) in the first few hours after the addition of trypsin to a concentrated tryptic digest. Both Henriques and Gjaldbäk and Wasteneys and Borsook failed to obtain any indication of synthesis when commercial trypsin was added to a tryptic digest. In view of the apparent dual action of commercial trypsin demonstrable in concentrated peptic digests it is possible that the discrepancy between the results of Bayliss and of Marston on the one hand, and of Henriques and Gjaldbäk and of Wasteneys and Borsook on the other is due to the observations of the former workers having been made in the first few hours after addition of the trypsin, while the latter workers confined their observations to much later periods.

The one hitherto unchallenged synthesis of protein by trypsin from a tryptic digest is that reported by Taylor (24) (25) in 1907 and 1909. The enzyme employed here, however, was not "trypsin" in the sense that it was derived from the pancreas, but it was the glycerol extract of the liver of the Pacific coast clam (Schizothaerus Nuttalii). The synthesis achieved by Taylor was effected by a hepatic proteolytic enzyme, a member of the class of the autolytic enzymes, rather than by a true trypsin. When an extract of pancreas was added to the concentrated solution of amino acids isolated from a tryptic (hepatic) digest of protamine sulphate, a precipitate was obtained which was different from that produced when the extract of liver was added. The nature of the precipitate is obscure and it is doubtful whether it is a protein.

The synthesis obtained by Taylor resembles those effected by various tissue enzymes, i.e., autolytic enzymes. In 1903 Nürnberg (26) found that extracts of liver brought about precipitates in solutions of proteoses.

Extracts of stomach, lung, pancreas, small intestine, large intestine, kidney, brain, egg and muscle also possess this property. The precipitates produced by these extracts corresponded in amount to the order in which they are given above. Nürnberg's observations were confirmed by Grossman (27) (28).

Abderhalden (29) reported observations of reversion of autolysis in concentrated autolysates when the enzyme preparation employed consisted of an extract of the same tissue as that from which the digest was prepared. He allowed a tissue to autolyze for some three months. It was then concentrated, a fresh extract of the same tissue was added and the mixture was set away at room temperature for five months. Measures were taken to ensure the sterility of the mixture. Indications of reversion were obtained with liver, thyroid, lung and kidney. No reversion was obtained in an autolysate of yeast, nor when an extract was added from a tissue other than that which served to form the digest. Kostytchew and Brilliant (30) claimed to have obtained reversion in a yeast autolysate by adding 40 per cent of glucose. They employed precipitability by lead acetate as a criterion of synthesis. When precipitability by copper hydroxide was employed the results were negative. Borsook and Wasteneys (31) could obtain no evidence of synthesis under the conditions described by Kostytchew and Brilliant using precipitability by 2 per cent trichloroacetic acid, or reduction in the formol titration values, or fractional analysis as criteria (32). Zak (33) claimed to have observed a slow synthesis of secondary proteose at the expense of material not precipitated by phosphotungstic acid in unconcentrated filtrates of B. pyocyaneus grown in bouillon. These filtrates were kept for 20 to 30 days at incubator temperature.

In most of the experiments mentioned above the visible effect of the presence of enzyme in a concentrated digest was the appearance of a precipitate. This precipitate, it was taken for granted, was more complex than any of the constituents of the digest before addition of the enzyme. In support of this surmise it was pointed out that the precipitate occurred only in concentrated solutions, and that it did not occur if active enzyme was not added. No further proof of the protein nature (as distinguished from proteose or peptone) of the precipitate was presented, although some observers investigated the phenomenon more intensively.

Okunew (3) evaporated digests to dryness before and after the action of added pepsin and estimated that there was 2 per cent more water after the action of the enzyme than before. This was quoted as evidence that

plastein formation is an anhydride formation, i.e., the reverse of hydrolysis. Sawjalow, discussing Okunew's results, believed that plastein was a synthetic product because of its property of forming gels in concentrated solutions of products of peptic digestion; and because of its greater precipitability by metallic salts. Glassner (34) fed animals with meat and killed them after some hours, he then removed the stomachs and scraped off the mucous membrane. Half was boiled, the other half was incubated at 40° for three hours. He observed that in the unboiled solutions, as compared with the boiled, the proteose diminished; while certain insoluble material, which may have been protein, increased. The subproteose fraction appears to have remained constant. Herzog (35) like Sawjalow pointed to the increase in viscosity during plastein formation as evidence of synthesis.

In 1907 Sawjalow (36) attempted to estimate the "order" of the reaction. He measured the time required to form a given amount of plastein. He argued that if the reaction was monomolecular the amount formed in a given time would be proportional to the concentration; if bimolecular it would be inversely proportional and if trimolecular inversely proportional to the square of the concentration. The results obtained indicated, according to Sawjalow, that the reaction was bi- or trimolecular. But as later investigations showed, the reactions were carried out at suboptimal hydrogen ion concentration. The "constants" varied considerably, but Sawjalow maintained that regardless of their variability the fact that they never agreed with monomolecular, but sometimes went beyond tri-molecular values proved that the reaction was a synthesis. The same author also attempted to determine the base combining power. Titrating to a rose colour with phenolphthalein he found the average equivalent weight for plastein to be 6000, and only 3000 for proteose. His material also appeared to be heat coagulable.

None of this evidence is unassailable. Sawjalow, however, made two observations of greater significance. He found, using Mett's tubes, that albumin is not digested in concentrated solutions of proteose; and that plastein suspended in acid solution is digested by pepsin.

In 1907 Robertson (8) (12) obtained a precipitate in a concentrated solution of the products of peptic hydrolysis of casein under conditions similar to those in which plasteins are obtained. Robertson believed that the precipitated substance was "paranuclein," one of the most complex products appearing during the early stages of the peptic digestion of casein and which disappears, due to further digestion, in the course of the hydrolysis. His reasons for believing this precipitate to be a para-

nuclein synthesized from the less complex constituents of the digest were based on the following evidence. The precipitated substance was insoluble in acids and readily soluble in dilute alkali. It precipitated protamin from a 1 per cent solution in a reaction just alkaline to phenolphthalein. A 2 per cent solution of the precipitate in N/100 NaOH was precipitated by M/10 ferric ammonium sulphate. These are properties of paranuclein and not of the simpler products of the peptic digestion of casein which alone constituted the concentrated digest in which the paranuclein was formed. The phosphorus content of the synthesized substance was 1.61 per cent; that of hydrolytic paranuclein, separated in the first stages of digestion of casein, is, however, 4.18 per cent. Robertson claimed that the latter paranuclein was a mixture and that one of its constitutents contained 1.51 per cent P_2O_5 which corresponded with that of the synthesized material. Robertson also pointed to the elementary constitution and to certain immunological reactions as evidence of synthesis.

Robertson's conclusions were contested by Bayliss (22) (37). Bayliss believed the precipitation observed by Robertson to be, not a result of enzyme action, but a colloidal precipitation. Bayliss stated that the reaction did not occur when the preliminary digestion of the casein was carried out in a more acid reaction, i.e., nearer to the optimum reaction than was the case in Robertson's procedure. The reaction also failed if the digest was filtered before concentration. Bayliss stated further that not all pepsin preparations gave the reaction observed by Robertson, and that pepsin, whose hydrolyzing power is lost in alkaline solution, still possesses the power of forming this precipitate. On the other hand, according to Bayliss, pepsin which has been allowed to stand for several days loses its "synthesizing" power but retains most of its hydrolyzing power.

The objections raised by Bayliss against Robertson's claims that the precipitate formed in his experiments was a synthetic product were not substantiated by the results of Wasteneys and Borsook. Their observations, though made on digests of albumin, instead of casein, confirmed in principle most of the conclusions of Robertson. It must, however, be pointed out that while his comparisons of the elementary analyses show differences between hydrolyzed paranuclein and the synthetic product, identity in elementary composition is no proof of synthesis because the elementary composition of proteoses, themselves substances of high molecular weight composed of many amino acids, differs little from that of the proteins. The precipitation reactions, though more sat-

isfactory, cannot be taken as convincing proof that the substance is a protein because it is not possible to dismiss the objection that mere denaturation of the proteoses, without any synthesis, might account for most of the properties of the precipitated substance and that the properties of paranuclein may be due to this denaturation and may bear no relation to chemical complexity.

Henriques and Gjaldbäk (13) (14), in 1911, furnished the first unequivocal evidence that plastein formation by pepsin is synthesis, by demonstrating that there is always a decrease in the free amino nitrogen in a digest after plastein formation. Since the increase of the free amino

TABLE 2

The ratio of free amino to total nitrogen in native and synthesized proteins and their cleavage products

	$\frac{\text{FREE AMINO N}}{\text{TOTAL N}} \times 100$
Edestin*	2.8
Egg albumin*	6.7
Casein*	11.9
Fibrinogen*	7.1
Serum globulin*	6.0
Witte's peptone*	14.4
Plastein from peptic digest of egg albumin**	6.6
Egg albumin**	3.5
Proteose from peptic digest of egg albumin**	10.5
Peptic digest of plastein**	17.5

* Values obtained by Henriques and Gjaldbäk employing formol titration.

** Values obtained by Wasteneys and Borsook employing Van Slyke method.

nitrogen of a protein digest has often been taken as a measure of the degree and rate of hydrolysis, a decrease in the ratio of free amino to total nitrogen may justifiably be interpreted as proof of synthesis. This criterion may not however be employed unreservedly. The ratio for casein is 11.9, while proteose derived from egg albumin has been found to give a value of 10.5. It is improbable, in spite of these values that casein is of lower complexity than a product of peptic digestion of egg albumin, viz, proteose. However in estimating the result of changes in the digest, or in comparing two substances isolated from the same digest, differences in the value of this ratio must indicate corresponding differences in complexity and this reservation is then unnecessary.

Henriques and Gjaldbäk determined the ratio of free amino to total

nitrogen in the isolated and purified plastein and compared it with the similar values of native proteins and protein split products. Wasteneys and Borsook confirmed their observations. Some of these results are collected in table 2.

It was found later that the ratio of free COOH groups to total nitrogen also was of the same order of magnitude in plastein as in native protein (38).

The reduction in free amino nitrogen was contested by Oda (39). He carried out his observations in a micro Van Slyke apparatus on the whole digest, but the amount of material that can be employed in this apparatus is so small that the reduction occurring is just barely detectable, being represented by 0.05 cc .of nitrogen gas when Oda's concentrations of material are employed. Later Rona and Chrometzka (40), while confirming the reduction of free amino nitrogen, found only indefinite variations in the carboxyl groups of the digest after peptic plastein formation. They accordingly expressed doubts regarding the synthetic nature of plastein and believed that the reduction in free amino nitrogen was due to deamination with the formation of ammonia. Later, however, Rona and Oelkers (41) found that the ammonia formation claimed by Rona and Chrometzka and the irregularities in the carboxyl group values were due to methodical errors. They found that the decrease in free amino nitrogen as determined by the methods of Van Slyke and of Linderstrom-Lang (42), were paralleled by exactly equivalent reductions in the free carboxyl groups as measured by the method of Wilstätter. They revised the opinion expressed previously by Rona and Chrometzka and accepted the view that peptic plastein formation is a synthesis.

The rapid hydrolysis of plastein by pepsin still further justifies the inclusion of this artificially synthesized product among the proteins. Hydrolysis of plastein in dilute solutions was observed by many of the early workers, particularly by Sawjalow and by Henriques and Gjaldbäk. Wasteneys and Borsook measured the rate of its hydrolysis by pepsin, and found that it is digested at the same rate as native protein and that it similarly yields proteoses and peptones among the products. Proteose salted out from the digest from which the plastein is synthesized cannot be digested by pepsin. The hydrolysis of plastein by pepsin, yielding proteoses, is evidence that its complexity is higher than that of proteose.

Proof of a different nature that plastein formation is a synthesis is afforded by the results of fractional analysis (15) of samples of a digest in which different amounts of plastein have been formed.

The analyses show, table 3, that all fractions of the peptic digest contribute to the formation of plastein, and in approximately the same relative amounts as they are obtained in the peptic hydrolysis of albumin.

Further proof of the protein nature of plastein is yielded by its precipitation by trichloroacetic acid. Plastein is precipitated from its solution in either concentrated or dilute solutions of strong acid, by 2 per cent trichloroacetic acid. In view of inability of this reagent to precipitate gelatine, which approximates protein closely, or any of the proteoses, there can be little room for doubt that peptic plastein is a synthetic product, and that its complexity is of the order of native protein.

The evidence for synthesis, in the case of the action of pancreatic trypsin on concentrated tryptic digests, is less convincing. The obser-

TABLE 3

Fractional analyses of peptic digests in which various amounts of plastein have been synthesized

Per cent of total nitrogen

PLASTEIN N	PROTEOSE N	PEPTONE N	SUBPEPTONE N
1.3	32.0	35.6	31.0
3.2	32.8	33.2	31.0
7.1	30.5	32.8	29.8
17.2	24.2	29.2	29.4
18.7	23.0	29.4	28.7
27.3	19.6	28.1	25.0

vations of Bayliss and of Marston, from which they concluded that synthesis had occurred, were not continued beyond the first few hours after the addition of the trypsin. Henriques and Gjaldbäk, and Wasteneys and Borsook, made their observations after 24 and 48 hours, and failed to obtain any evidence of synthesis. As suggested earlier, the discrepancy may be due to this difference in the times for which the trypsin was permitted to act.

Taylor's synthesis (24) (25), is unequivocal. The substrate he employed was a digest of protamine sulphate completely hydrolysed by a glycerine extract of clam liver. On the addition of more glycerine extract to a concentrated solution of this digest a substance was precipitated which his tests showed to be identical with the original protamin sulphate. This precipitate settled out gradually, over a period of 5 months. It was redissolved in acid and reprecipitated 4 times, and

the yield from 4 litres of digest was 1.8 gram. The solubilities of this substance were the same as those of protamin sulphate. In another experiment a concentrated solution was made of the amino acids isolated by the ester method from a digest of this protein, and extract of clam liver added. After some months a precipitate was obtained with the same properties as that obtained from the "tryptic" digest of protamin sulphate. On subjecting this precipitate to elementary analysis the following results were obtained.

	FOR $C_{16}H_{29}N_5O_2 \cdot H_2SO_4$	
	Theoretical	Observed
C	40.25	40.3
H	6.5	6.7
N	26.41	26.2
H_2SO_4	20.55	20.7

These agree closely with the results from protamin sulphate. This is the only enzymatic synthesis of protein from amino acids which has been reported.

A number of workers, as mentioned earlier, obtained precipitates in concentrated protein hydrolysates on the addition of various tissue extracts. These precipitates have been considered to be synthesized products obtained through the agency of the enzyme added, but the experiments reported by Abderhalden are the only ones in which any evidence is submitted in support of this assumption. In Abderhalden's experiments an organ was macerated, suspended in water and allowed to autolyze until the digests gave negative biuret tests and contained no heat coagulable material. The digests were concentrated and extracts of various tissues were added. The evidences of synthesis were the appearance of a heat coagulable material, of a positive biuret test, a reduction in the free amino nitrogen, and a diminution in the colour on testing for free tryptophane, which colour was regenerated after re-hydrolysis of the compounds synthesized. These evidences of synthesis were obtained whenever the enzyme added consisted of an extract of the same organ as that from which the concentrated autolysate was obtained; but no synthesis occurred when autolysate and enzyme extract were not derived from the same organ. Liver, thyroid, lung and kidney extract were employed. A concentrated autolysate of yeast, however, failed to give any evidence of synthesis when fresh yeast juice was added to it.

The results of Taylor and of Abderhalden are suggestive, but their biological significance is lessened by the paucity of the yields, and by the length of time required for these yields.

The chemical evidence supporting the thesis that the precipitate obtained after pepsin is added to a concentrated peptic digest, is the product of a synthesis, is augmented by some convincing biological evidence.

Beard (44) found that growth can be maintained as well on plastein synthesized from a peptic digest of egg albumin, as on the egg albumin itself. Gay and Robertson (43) found that paranuclein, and synthetic paranuclein synthesized by the action of pepsin from the products of a complete peptic digestion of casein, are interchangeable when tested by anaphylaxis and alexin fixation reactions. They possess identical and specific antigenic properties that are not present in the original peptic digestion products. Bayliss believed that these results were due to a "nuclein" which Robertson called synthetic paranuclein, present in the precipitate, and also in the native paranuclein. The failure of the digest to give this reaction, Bayliss believed, was due to the fact that the concentration of "nuclein" in the unconcentrated digest was too low. Since traces only are necessary for anaphylaxis antigens this criticism of Bayliss can hardly be considered valid. In any event this criticism certainly does not apply to the work of v.Knaffl-Lenz and Pick (45). These authors found that whereas peptone or the products of peptic digestion are extremely toxic when injected into the blood stream, plastein obtained by the action of pepsin on these products was non-toxic, even when as much as 1.8 gram was injected. On the intravenous injection of a small amount of a peptic digest the typical syndrome of "peptone shock" is evoked, vasodilatation of the splanchnic vessels, fall in blood sugar, reduction in the coagulability of the blood, and bronchial spasm. These symptoms produced by an injection of peptone, by a peptic digest, and not by plastein, are produced by the products of the peptic digestion of plastein itself. This is conclusive evidence of the higher complexity of plastein.

Chemical and physical properties of synthesized proteins. Plasteins prepared by the action of pepsin on a concentrated peptic digest possess similar general properties regardless of the protein which is the source of the digest. The properties of the plastein synthesized from a peptic digest of egg albumin are typical. It is only very faintly soluble at neutrality, but is quite soluble in dilute strong acids and alkalies, more so in the latter than in the former. A region of distinct solubility exists between pH 8.8 and 9.9, with a maximum solubility at pH 9.2. At

hydrogen ion concentrations somewhat greater than pH 1.0 it is fairly soluble, but is practically insoluble at all hydrogen ion concentrations between pH 2.0 and pH 7.0. It is only slightly soluble in 10 per cent sodium chloride, but is very soluble in strong nitric and hydrochloric acids. It is not soluble to any noticeable extent in 95 per cent alcohol. It is incompletely precipitated from its solution in 0.025 N sodium hydroxide by the addition of sodium chloride to half saturation at room temperature; a much heavier precipitate is obtained on saturation. Boiling coagulates plastein in alkaline, but not in acid solution. It is precipitated from its solution in either strong or weak hydrochloric acid by trichloroacetic acid. It gives a protein biuret, and gives a positive Heller's test. It is rapidly digested by pepsin at pH 1.7.

Sawjalow (46) observed that plastein was soluble in concentrated peptic digests at room temperature. When pepsin is added to a concentrated peptic digest of fibrin and the mixture is maintained at room temperature, the solution becomes gelatinous at the end of 24 hours. When this solution is neutralized and boiled there is a voluminous precipitate of coagulated protein. This precipitate, unlike heat-coagulated protein, resembles plastein. It is soluble in dilute acid and alkali and is insoluble at neutrality. Boiled in a faintly alkaline solution a voluminous, flocculent precipitate forms. The plastein is precipitated in the digest at room temperature after 24 hours. Sawjalow believed that plastein is formed as a soluble product but is subsequently rendered insoluble as a result of denaturation. The following observations (47) support this opinion. When a concentrated digest containing pepsin is poured into a wide, flat dish and left uncovered, the suspension, opaque and creamy on account of the insoluble plastein present, gradually becomes clear and transparent. On the addition of a little water the opacity reappears. The same phenomenon is noticeable during the first few minutes of synthesis in a concentrated solution of proteose. The mixture remains quite clear, but the occurrence of synthesis is demonstrable by the formation of a precipitate on the addition of trichloroacetic acid or on diluting with water, and by the formation of a coagulum on heating quickly to boiling.

The following observations (15) further tend to show that the insolubility of plastein is due to a denaturation resultant upon the conditions in which it is formed; i.e., that it is not a property of the substance when first synthesized. When a solution of albumin is added to a concentrated peptic digest at pH 4.0, a precipitate immediately forms which increases on standing; at the end of 1 hour all of the albumin is precipi-

tated. This precipitate is quite insoluble in water, and exhibits the same solubilities as plastein. It seems that soluble protein on coming into contact with a solution of products at pH 4.0 is immediately denatured, or at any rate rendered insoluble. Albumin cannot exist in the soluble form under the conditions in which peptic synthesis occurs. This precipitation is not complete at 38°C. at acidities less than pH 5.8 or in concentrations of peptic digest less than 5.0 per cent. At pH 7.0 albumin remains in solution in concentrations of peptic digest as high as 30 per cent.

The constitution of the plasteins, from the very nature of the conditions under which they are synthesized, cannot be as constant as that

TABLE 4

Elementary composition of plasteins (Sawjalow)

ORIGINAL PROTEIN OF PEPTIC DIGEST	PER CENT					
	C	H	N	S	O	S + O
Almond globulin	55.2	7.5	16.5	—	—	20.7
Almond globulin	54.9	7.1	15.0	—	—	23.0
Ovalbumin	55.7	7.5	15.3	—	—	21.4
Ovoglobulin	56.0	7.6	14.8	—	—	21.6
Peptone (Gehe)	54.6	7.4	14.7			23.4
Peptone (Witte)	55.5	7.4	14.7	1.1	21.3	22.4
Serum globulin	54.7	7.5	14.8	—	—	23.0
Serum albumin	55.4	7.6	15.1	—	—	21.9
	56.8	7.6	14.6			21.0
Edestin (pumpkin seed)	55.4	7.9	15.2	1.4	20.1	21.4
Edestin (hempseed)	—	—	15.7	—	—	—
Myosin	54.9	7.1	14.7	1.2	22.1	23.3
Casein	55.7	7.2	14.9	1.2	21.2	22.4

of native protein. Apart from the presence in any given proteose or peptone of a radicle which will permit of its being linked to another fragment, there can hardly be any selection in the combination of the protein cleavage products to form a molecule of plastein. The chance meetings of the enzyme and synthesizable fragments determines the constitution of the plastein. The elementary constitution of such molecules can represent statistical averages only, and not constants characterizing a chemical individual. No evidence can be deduced regarding the complexity of the plastein molecule from the elementary constitution. The elementary constitution of a proteose or peptone can be little different from that of protein.

The elementary analyses of Sawjalow (36) are shown in table 4. Sawjalow's figures agree substantially with those of Wait (48), Lawrow (49), Kurajeff (18) (50) and Rosenfeld (51).

Robertson and Biddle (55) found that paranuclein synthesized at 60°C. had the same elementary composition as paranuclein isolated from an early peptic digest, but "paranucleins" synthesized at 36° had a higher carbon content, which they believe may have been due to irremovable toluol contamination.

Taylor's synthetic protamin, prepared from a concentrated solution of amino acids, corresponded in its elementary composition, within experimental error, exactly with the native variety. It was soluble in 35 parts of water, was precipitated by 3 parts of alcohol, was salted out by 10 per cent sodium chloride, was digested by trypsin and resisted the action of pepsin.

TABLE 5

Amino acid composition of plastein (from fibrin) and of fibrin (Levene and Van Slyke)

	100 GRAMS PLASTEIN	100 GRAMS FIBRIN
	grams	*grams*
Tyrosin	3.03	3.1
Glycocoll	0.5	2.2
Alanin	?	3.1
Valin and leucin	15.59	13.0
Phenylalanin	1.0	1.2
Glutamic acid	10.02	6.8
Aspartic acid	2.15	1.7
Prolin	2.55	2.4
Histidin	0.43	—
Arginin	2.06	—
Lysin	1.42	—
Tryptophan	Present	+

Levene and Van Slyke (52) determined gravimetrically the amino acid composition of plastein prepared from a peptic digest of fibrin (Witte peptone), and of fibrin itself (table 5). They also endeavoured (53) to estimate the complexity of the plastein molecule by comparing its viscosity with that of proteins and of proteoses. They found that solutions of all these substances showed a high initial viscosity which

diminished with time. The initial viscosity of plastein dissolved in normal alkali (which probably hydrolyzed the plastein to some extent) was nearer to proteose than to protein; but later the viscosity of the plastein fell to the same final level as that of the protein.

Herrmann and Chain (54) prepared plastein from Witte's peptone and found that it served as an antigen in the precipitin reaction not only for plastein from Witte peptone, but also for plasteins prepared from peptic digests of edestin, serum albumin, egg albumin, and almond globulin. Witte peptone did not evoke precipitins for Witte peptone or for casein. They argue from these observations that all the plasteins possess some common chemical structure.

Factors controlling protein synthesis. The first condition recognized as an essential prerequisite for protein synthesis is adequate concentration of the products of hydrolysis. The first observations of Danilewski were made on a concentrated peptic digest. Sawjalow (3) noticed that the precipitating action (i.e., plastein formation) progressively improved with increasing concentrations of products above 8.2 per cent; when the concentration of the digest was lower than 8.2 per cent no synthesis occurred. He noticed, further, that addition of products inhibited peptic hydrolysis and came to the curious conclusion from these results that peptic hydrolysis and plastein formation were brought about by two different enzymes.

A usual method of demonstrating the effect of the backward reaction is to add, at the outset, some of the products of the reaction. A retardation of the forward reaction, proportional to the amount of products added, is then observed. This effect may be demonstrated also for protein hydrolysis (55), (3). A. E. Taylor clearly recognized the significance of concentration and demonstrated it by the following experiment. A saturated solution of protamin sulphate was digested with trypsin, and as the digestion progressed, fresh portions of the powdered protein were from time to time added. It was found eventually that a fraction of the added protein remained undigested, even when fresh enzyme was added. If the solution was diluted then digestion began again. In Taylor's experiment and in experiments of Morrell, Borsook and Wasteneys (55) it was found that the rate of hydrolysis of protein, as measured by the amount digested in the first half-hour, diminishes as the initial concentration of protein increases. This is true whether soluble or coagulated egg albumin is employed as substrate. These observations demonstrate that the backward reaction is an important factor even during the course of active hydrolysis.

The importance of concentration in plastein synthesis was noted early. Grossmann (27), in 1906, found that if the concentration of the digest is not high enough no plastein formation occurs, but he did not define the limiting concentration. Lawrow (49) found that whereas albumin did not inhibit the coagulating action of rennet, it inhibited its ability to form plastein in a concentrated solution of products. Henriques and Gjaldbäk (14) observed an influence of the concentration of the digest on the yield, but they give no data. They observed also a rehydrolysis of the plastein on dilution of a digest in which plastein formation had occurred, when hydrochloric acid and fresh enzyme were added. They concluded that synthesis and hydrolysis are reversible processes depending upon the concentration of the reacting components.

The effect of concentration of products on peptic synthesis was studied in detail by Borsook and Wasteneys (17). They confirmed Moore's prediction that the important factor in the enzymatic hydrolysis and synthesis of proteins *in vitro* is the concentration of material in solution and not the relative concentration of water. They found that at low concentrations no synthesis takes place but, as the concentration increases, synthesis appears and increases with increasing concentration as shown in table 6. The crucial concentration where no synthesis occurs at 37°C., is in the neighbourhood of 7 per cent of products. Considerable change in enzyme concentration has only little effect upon the position of this limiting concentration. Above this concentration there is a straight line relationship between the amount of protein synthesized and the concentration of material in solution. At high concentrations, however, there is a rapid falling off from this relationship, until with progressively higher concentrations the yield quickly diminishes to zero. The diminishing yield sets in when the concentration rises above 40 per cent. The concentration of water under these conditions apparently begins to be significant. A related phenomenon was encountered by Armstrong and Gosney (56) in their experiments on the enzymatic synthesis of fats. They attributed the inhibitory influence of too low concentrations of water to the resulting diminution or removal of the surfaces in the heterogeneous systems of oily substances in water with which they were dealing. In their own words "Apparently, the intervention of a film of water is necessary at the interface of the system, where interaction takes place; if this be removed, action comes to an end."

Synthesis does not take place at 38°C. if the hydrolytic products are less concentrated than 7 per cent (table 6). When, however, synthesis is carried out in a much more concentrated solution, equi-

librium is attained at a point where the concentration is higher than 7 per cent even though the product of the reaction, protein, appears to be precipitated and hence removed from the reacting system, as soon as it is formed. This is shown in table 7. The explanation seems to lie in the fact that the protein synthesized remains in solution and is not precipi-

TABLE 6

Effect of substrate concentration on synthesis at 38°C.

ENZYME CONCENTRATION CONSTANT			ENZYME CONCENTRATION VARYING		
Relative concentration of digest	Relative concentration of enzyme	Protein synthesised	Relative concentration of digest	Relative concentration of enzyme	Protein synthesised
		mgm. of N			*mgm. of N*
10	10	210	10	10	200
8	10	140	7.5	7.5	100
6	10	80	—	—	—
5	10	50	5	5	60
4	10	50	—	—	—
3	10	40	3	3	10
2	10	10	2	2	0
1	10	0	1	1	0

TABLE 7

Equilibrium concentrations of synthesised protein and unsynthesised products for various initial concentrations of products

AMOUNT OF PROTEIN FORMED	CONCENTRATION OF SUBSTRATE BEFORE PROTEIN FORMATION	CONCENTRATION OF SUBSTRATE AFTER PROTEIN FORMATION HAS STOPPED
grams per cent	*grams per cent*	*grams per cent*
11.9	50.3	38.4
3.9	26.7	22.8
1.5	18.7	17.2
0.7	14.7	14.0
0.5	12.7	12.2
0.4	10.6	10.2
0.1	8.9	8.8

tated, after it is synthesized. Plastein is fairly soluble in concentrated, as distinguished from dilute, solutions of the products of hydrolysis. If previously re-synthesized protein is added to a concentrated digest with pepsin, subsequent synthesis is inhibited to an extent directly proportional to the amount added (17) (fig. 1). This proves that precip-

itated plastein takes part in the equilibrium as if it were in solution. It is the complement of the experiment where addition of products to a digest in which hydrolysis of protein is ensuing, inhibits hydrolysis to an extent proportional to the amount of products added.

In the theoretical discussion it was pointed out that the effect of temperature on the equilibrium position is such that increase of temperature results in a higher equilibrium yield of protein, while a decrease in temperature diminishes the equilibrium yield. In other words increase in temperature favours synthesis and decrease favours hydrolysis. Such effects were observed by Sawjalow (3), and Henriques and Gjaldbäk (14); but their investigations do not seem to have extended beyond qualitative observations. Robertson (8) measured the yields at

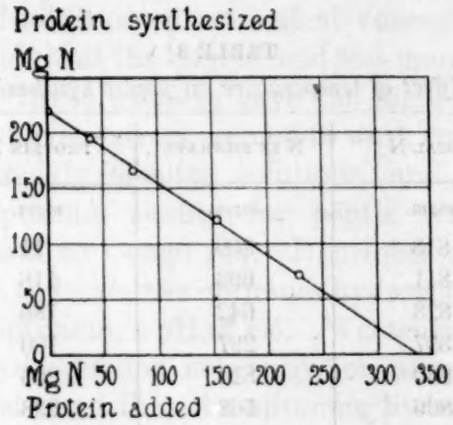

Fig. 1. The effect of the addition of previously synthesized protein on subsequent synthesis.

various temperatures and concluded that increase in temperature shifted the equilibrium more and more toward the protein side of the reaction because changes in temperature affect unequally, hydrolyzing and synthesizing components of the enzyme which he postulated. Roberston states that synthesis occurs at temperatures at which the hydrolyzing component is known to be destroyed. He quotes this effect of temperature as evidence for the existence of separate hydrolysing and synthesizing components in pepsin, but it should be mentioned that, while commercial pepsin is destroyed at temperatures above 45° in the absence of substrate, in the presence of substrate it withstands temperatures as high as 72° for some time (9). The highest yield of plastein is obtained at 72°C. At 80°C. no synthesis occurs due to immediate destruction of the enzyme. These results are shown in

table 8. Borsook and Wasteneys state that neither by preliminary solution in alkali nor by heating is it possible to effect any separation of synthesizing and hydrolyzing components of the commercial pepsin employed. Their experiments also do not confirm the result obtained by Bayliss (22) (37), and quoted by him in criticism of the experiments of Robertson, that alkali, which destroys the hydrolytic activity of pepsin does not remove the precipitating (i.e., synthesizing) power of the enzyme. Bayliss, in discussing Robertson's results, suggested that the synthesis (precipitation) described by him, was probably due to the particular sample of pepsin employed. It may be worth pointing out, however, that plasteins have been obtained by Danilewski and other Russian workers by means of both animal and fish gastric juice, that Robertson employed Grubler's pepsin, while Wasteneys and Borsook

TABLE 8

Effect of temperature on peptic synthesis

TEMPERATURE	TOTAL N	N IN FILTRATE	PROTEIN N	PROTEIN N OF TOTAL N
°C.	mgm.	mgm.	mgm.	per cent
13	818	809	11	1.5
21	811	693	118	14.5
30	828	642	186	22.4
37	397	297	100	25.3
49	830	824	206	24.9
61	806	568	238	29.5
72	823	576	247	30.0
80	823	823	0	0

have employed many different batches of Eimer and Amend, and of Merck pepsin.

For the inseparability of the hydrolyzing and synthesizing components there seems no necessity for postulating two enzymes or two components of one enzyme, the one functioning in hydrolysis and the other in synthesis. Assuming only the classical function of a catalyst, a shift in equilibrium is accounted for on thermodynamic grounds, when the only influence ascribed to the enzyme is the acceleration of the attainment of the point of equilibrium, wherever that point may be defined by the heat, chemical energy, and osmotic pressures of the reacting components. The effect of temperature is easily demonstrable in the case of proteins because as Hari (7) and von Lengyel (6) showed, the heat of reaction is practically zero. The backward reaction, whose influ-

ence is demonstrable even when hydrolysis is in progress, is affected by temperature in the same manner as it is in concentrated solutions of products where synthesis is in progress; i.e., its retarding influence is increased. This is the case whether the protein hydrolysis is a soluble protein, or an insoluble one like coagulated albumin.

Most of the work on enzymatic synthesis was carried out before the present ideas regarding the importance of the hydrogen ion concentration in enzyme reactions had been developed. The absence of any control of this condition in all earlier work is the probable cause of the many discrepancies in the work of different observers. Sawjalow recognized the importance of acidity. He found that the yield varied with the amount of acid added to a concentrated digest and that peptic synthesis failed completely in 0.5 per cent sodium carbonate. He concluded from the results of addition of equivalent concentrations of various strong and weak acids that the kind of acid was immaterial. Nurnberg (26) observed that tissue extracts gave the highest yields in weakly acid solutions. But liver extract seemed to work equally well in weakly acid, neutral, or weakly alkaline solutions, and Lawrow (29) observed that the optimum acidity for peptic synthesis occurred in solutions weakly acid to Congo red. Henriques and Gjaldbäk were the first to attempt to locate the optimum hydrogen ion concentration. They gave, as the optimum, a pH of 1.5. Wasteneys and Borsook (16) found that this hydrogen ion concentration allows only very small amounts of synthesis, and that the optimum hydrogen ion concentration is at pH 4.0. Oda (39) confirmed this and found further that the optimum was the same regardless of the acid employed. He found also that the yield at the optimum was not affected by the presence or absence of such salts as NaCl, KCNS, KF, KBr, KCN, NaF, KI, KNO_3, K_2SO_4, KCl and $CaCl_2$.

If a curve is constructed of the yields obtained at various hydrogen ion concentrations with the per cent of the yield at the optimum (which is taken as 100 per cent) plotted as ordinates and with pH plotted as abscissae, the curve resembles in form and slope either the primary dissociation curve of a dibasic acid or the curve representing the undissociated residue of an amphoteric electrolyte. The dissociation constants derivable from this curve do not correspond to the dissociation constants of any of the known amino acids. It would be surprising if any such correspondence existed, for conditions in the digest, or in the mode of combination of the amino acid, modify the values of the dissociation constants. Because of the correspondence between experimental

values and a theoretical dissociation curve, and because titration curves show that the ionization of the digest taken as an entity is not represented by the curve, it seems probable that the amount of protein synthesized at any given C_H is dependent upon the degree of ionization of one component of the digest, i.e., the substrate.

The effect of varying the hydrogen ion concentration might, on the other hand, be attributed to variations brought about in the functional condition of the enzyme. There are two possibilities; the varying acid-

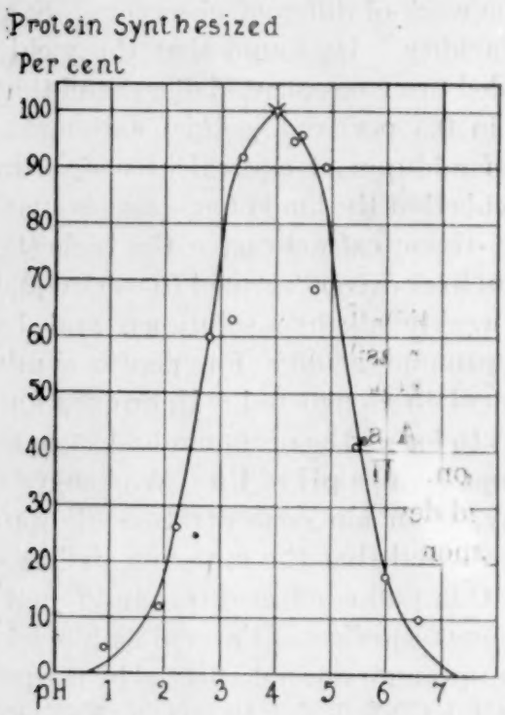

Fig. 2. Relation between pH and amount of protein synthesized. The curve is a theoretical dissociation curve. Points are experimental values.

ity affects either the rate of autodestruction of the pepsin or its ionization. If autodestruction is the variable factor, then the optimum pH is that degree of acidity in which synthesis proceeds for the longest time before it is brought to a stop by the disappearance of active enzyme. Michaelis and Rothstein (57) and Morgenroth (58) found that in 10 per cent sodium chloride, pepsin solutions at approximately pH 3.3 may stand at room temperature for a week without suffering any autodestruction and in glycerol for more than a year. In simple aqueous solutions autodestruction does not proceed at any significant rate until the reac-

tion of the solution approximates pH 6.0 (59). The alternative possible effect of acidity, viz., its influence upon the degree of ionization of the enzyme, is more difficult to eliminate. It was observed that the relationship between the equilibrium amount of synthesis and concentration of enzyme is not linear but exponential of the type $Y = Ax^n$ (16). If the curve obtained from the relationship between hydrogen ion concentration and amount of synthesis, which has the form of a dissociation curve, represents the dissociation of the enzyme, then a direct linear relationship is obligatory between concentration of active enzyme and velocity of synthesis. No experiments are available for a decision on this point. Northrop (67) has shown, however, that between pH 1 to 7 pepsin exists as a monovalent anion and that changes in pH within that range do not affect the degree of its dissociation. It is not, therefore, through any change in the state of the enzyme that varying acidity affects synthesis.

Contrary to classical conceptions of enzyme action it is found that the equilibrium position in synthesis is definitely affected by the concentration of enzyme. Sawjalow found that the amount of synthesis in 24 hours increased with increasing concentration of enzyme up to a certain concentration, above which further addition of enzyme no longer induced a higher yield. A similar effect of enzyme concentration was observed by Robertson. He concluded that the equilibrium quantities of protein synthesized depended in part on the concentration of pepsin, though in his experiments velocities only were measured. Robertson's conclusions are confirmed by Wasteneys and Borsook's measurements of equilibrium amounts for different pepsin concentrations. If the true equilibrium position of the system can be represented by the final amount synthesized, then these experiments furnish an exception to the general "law" of enzyme action and catalysis, which states that the equilibrium position is not affected by the concentration of the enzyme. Certain results, to be discussed later, may throw some light on this contradiction. It is possible to consider that pepsin owes its action, in part at least, to properties resembling those possessed by emulsifying agents. Certain emulsions, in concentrated digests, accelerate the attainment of equilibria. In the presence of some emulsions synthesis is effected even in the absence of enzyme. This action of emulsifying agents occurs by virtue of the interphase surface. There is a similarity between the action of pepsin and of emulsions in that increasing amounts of these agents, under certain conditions, augment the yield of protein synthesized at equilibrium. This effect of concentration of enzyme, or

emulsifying agent, on the equilibrium point is consistent with thermodynamical conceptions because addition of emulsifying agent means an increase in the interphase surface, and the increase in interphase surface means an addition of energy to the system because of the increased amount of surface energy available. Increasing the concentration of pepsin should, then, have the same effect as that obtained when energy in any other form is added to the system as, for example, by increasing temperature. The fact that increasing the concentration of enzyme has the same effect as increasing temperature, may be considered as evidence somewhat supporting the above hypothesis regarding the mechanism of the action of the pepsin.

Synthesis does not proceed equally well in all types of protein digests. A concentrated peptic digest of albumin may yield as much as 50 per cent synthesis, whereas similar digests of gliadin (47) under comparable conditions yield only 10 per cent. Peptic digests of gelatin have, so far, failed to yield any plasteins (60) (14) (15). Only slight yields, if any, are obtained in incomplete acid or alkaline hydrolysates with either pepsin or trypsin (14) and concentrated solutions of amino acids do not readily support synthesis (61).

The synthesis most studied has been peptic synthesis in concentrated peptic digests. In these digests many fractions, including a great variety of proteoses, peptones and polypeptides are present, and it is important to ascertain whether synthesis is restricted to specific fractions. A great deal of work has been done in an attempt to discover the relative significance of the various fractions of the digest in plastein formation. As stated earlier, in practically all of the early work on protein synthesis, the importance of the hydrogen ion concentration was ignored, and the importance of substrate concentration was fully recognized only by Sawjalow. The concentrations used rarely exceeded 11 per cent and were often as low as 3 per cent. No reliance can therefore be placed on the conclusions drawn from these experiments (3) (62) (50) (15) (26) (36) (60), although claims were made for most of the fractions into which these workers arbitrarily divided the digest, that they did or did not support synthesis. Lukomnik (63) found that the dialysate and the residue from Witte peptone both permitted plastein formation. Sawjalow (36) observed that a number of fractions of a peptic digest, none of which separately supports synthesis, yielded considerable crops of plastein on being joined. He observed further that a peptic digest of plastein contained all 4 types of proteoses, proto- and heteroproteose, α and β-deuteroproteose. From these observations he concluded that all

types of proteose participate in synthesis. Kurajeff (60) had shown previously that resynthesis could be obtained in a concentrated peptic digest of plastein. In this early work only positive results can be considered as significant. Negative results may have been due to the use of a hydrogen ion concentration far removed from the optimum, or more often to inadequate concentration. Under conditions where the hydrogen ion concentration is optimum and the concentration high enough to permit a large fraction of the digest to be converted to plastein, all fractions in a digest contribute to the formation of plastein, and in approximately the same proportions as they arise during hydrolysis, as table 2 shows. This observation is essentially the same as Lukomnik's. Isolated proteose and subproteose fractions of a peptic digest each give approximately the same yield of plastein, and this yield is the same as in a similar concentration of the whole digest (47). The plasteins so synthesized differ, however, in their number of carboxyl and amino groups from those plasteins obtained from the whole digest, and from those obtained from each other. The plasteins synthesized from the fractions contained more free carboxyl groups and amino groups. As stated above, Lawrow had observed that the plastein synthesized from the filtrate of a digest in which plastein formation had occurred, differed in its elementary composition from a plastein synthesized from the original digest. It may be concluded therefore, that no one fraction of a digest contains all of the synthesizable material. On the other hand, not all of the products resulting from the peptic hydrolysis of protein are capable of re-synthesis. The results point to the presence or absence of a synthesizable group or complex in a given fraction which determines whether it is synthesizable or not. As already mentioned, peptic digests of gelatin do not support synthesis. From the fact that Henriques and Gjaldbäk found that partial acid or alkali hydrolysates yield no plasteins, it is possible that the failure to obtain synthesis in peptic digests of gelatine is due to the treatment with strong acid which is employed in converting collagen to a gelatine. Since gelatine can be hydrolyzed by pepsin, although a gelatine digest cannot be resynthesized, it seems impossible that the radicle which is split in hydrolysis is re-formed in synthesis. However, plasteins can be synthesized from fractions which are not hydrolyzed further by pepsin; and the plastein so synthesized is hydrolyzed by pepsin. These two sets of observations cannot, for the present, be reconciled with the hypothesis that the enzyme synthesizes in a concentrated solution the same linkage which it hydrolyzes in dilute solution.

Sawjalow (3) from qualitative experiments, concluded that the nearer a given fraction approximates in complexity the original protein the more suitably does it serve as a substrate for peptic synthesis. Henriques and Gjaldbäk (14) who used more exact methods, arrived at the same conclusion. They found that when a peptic digest is exposed to the continued action of pepsin, the ability of the digest to "gelatinize" when pepsin is added after concentration of the digest, diminishes. It diminishes the more, the longer the action of pepsin in the unconcentrated digest continues. They found, however, little change in the

TABLE 9

The effect of duration of peptic hydrolysis on the amount of plastein formed

DURATION OF HYDROLYSIS	DURATION OF SYNTHESIS	TOTAL N IN 5 CC. OF CONCENTRATED DIGEST	N OF SYNTHESIZED PROTEIN IN 5 CC. CONCENTRATED DIGEST
days	*days*	*mgm.*	*mgm.*
6	3	660	178
	11	662	198
	15	658	196
13	3	682	122
	11	674	152
	15	676	150
21	3	636	88
	11	654	132
	15	660	136
40	3	690	74
	11	684	88
	15	688	90

amount of plastein formed. Borsook, MacFadyen and Wasteneys reexamined this phenomenon, controlling the concentration of material in solution and the hydrogen ion concentration. They found that the longer the dilute digest was allowed to contain active pepsin the less was the subsequent yield of synthesized protein (table 9).

This progressive loss of synthesizability may be due to the pepsin or to the acid. It can be shown that the prolonged action of acid alone at $C_H+ = 10^{-1.6}$ does not reduce the synthesizability of the digest. It must therefore be concluded that the slow and prolonged secondary hydrolysis due to peptic action (66) is responsible for this phenomenon. From the fact that a 40 day digest as compared with a 6 day digest

affords at equilibrium less than half the yield of plastein, it may be concluded that not all of the material in the 40 day digest can take part in synthesis.

In any one digest the yield is controlled by the equilibrium position, nevertheless, when the plastein is removed further synthesis sets in. This was observed by Lawrow (49) who found also that plastein formed from the filtrate obtained when the "equilibrium" plastein is separated, differed in its elementary composition from that first synthesized. If protein synthesized from a concentrated digest is removed by filtration after dilution, and the filtrate re-concentrated, and its reaction re-adjusted to pH 4.0, synthesis may again be induced with pepsin. This may be repeated three times, so that at least four crops of protein are obtained from one original portion of concentrated digest (47). The maximum yield diminishes progressively. Obviously the cessation of synthesis at any one time is not due to complete utilization of all the material essential for synthesis. The diminution in the yield however shows that not all parts of the digest are equally synthesizable. The cessation of synthesis in any one digest is due to the attainment of equilibrium and not to the complete utilization of available synthesizable material. The amount of the equilibrium yield on the other hand is dependent on the amount of synthesizable material in the digest.

The influence of emulsions on peptic synthesis. In 1926 Robertson (58) discussed the possibility that concentration by orientation at lipoid surfaces, of the reactive groups of amino acids, might account for the completeness of enzymatic synthesis in living tissues. In this connection Marston (64) made the interesting suggestion that syntheses in the cell are located at the surfaces of mitochondria. Oda (39) in the same year reported that lecithin and cholesterol emulsions did not influence the yield of plastein to be obtained with pepsin from concentrated peptic digests of several proteins. Marston (23), with trypsin, observed an acceleration of the rate of synthesis in a tryptic digest when the lipoids of egg-yolk were added with the enzyme.

In peptic synthesis, when emulsions formed with benzene or its derivatives are present with the digest and enzyme, a marked acceleration of the rate of synthesis is induced (38). Among all the emulsifying materials tried benzene and benzaldehyde proved to be most effective. Chloroform is slightly efficient, xylol, talc, kieselguhr, and barium sulphate are less efficient, and fats and fatty acids are ineffective. Synthesis can be effected even without enzyme by the emulsifying agent alone if it is an accelerator of enzymatic synthesis (table 10). The yield with

emulsifying agents alone is sufficiently large in the cases of benzene and benzaldehyde to permit isolation and some characterization of the proteins so synthesized.

TABLE 10

The effect of emulsifying agents on the rate of synthesis in concentrated peptic digests of albumin

EMULSIFYING AGENT	SYNTHESIS IN 24 HOURS WITH ACTIVE PEPSIN	SYNTHESIS IN 24 HOURS WITH INACTIVE PEPSIN
	mgm. protein N in terms of 10 cc. of original digest	*mgm. protein N in terms of 10 cc. of original digest*
None	10	Nil.
Oleic acid	9	Nil.
Olive oil	9	Nil.
Barium sulphate	14	Nil.
Talc powder	15	Nil.
Kieselguhr	14	Nil.
Chloroform	19	Nil.
Benzene	38	8
Toluene	24	4
Xylol	16	1
Benzoic acid	25	4
Benzaldehyde	51	11

TABLE 11

Properties of proteins synthesized from a concentrated peptic digest by various methods

	SYNTHESIZED BY PEPSIN ALONE	SYNTHESIZED IN A BENZENE-DIGEST EMULSION	SYNTHESIZED IN A BENZALDEHYDE-DIGEST EMULSION	SYNTHESIZED IN A BENZALDEHYDE-PEPSIN-DIGEST EMULSION
Maximum base bound per gram (equivalents $\times 10^5$)	177	221	195	167
Nitrogen in air-dried protein (per cent)	13.6	13.7	13.9	12.4
Free amino nitrogen (per cent of total nitrogen)	8.4	—	5.9	6.4
Free carboxyl groups $\left(\frac{\text{N/14 equivalents} \times 100}{\text{Total nitrogen}}\right)$	10.5	—	12.6	6.0
Peptic hydrolysis (per cent in three hours)	56	—	6	75

These effects of emulsifying agents on the peptic synthesis of protein are probably examples of specific adsorption. The valencies involved, it seems, are secondary rather than primary in Langmuir's (65) interpretation of these terms. This is suggested by the efficiency of such inert chemical compounds as benzene, toluol, and chloroform, which under these circumstances obviously do not enter into chemical combination with constituents of the digest for, while they are incompletely removed by repeated washing with water, they are entirely separated by alcohol, leaving a protein which contains the same percentage of nitrogen as that synthesized by enzyme alone.

The chemical and physical properties of the synthesized proteins vary with the means employed in their synthesis, table 11. On comparing the combining capacities of proteins synthesized by pepsin alone, by pepsin plus benzaldehyde, by benzaldehyde alone and by benzene it is found that the protein synthesized by benzene alone possesses the highest base combining capacity. That synthesized by benzaldehyde alone has a similar but lower combining capacity while those proteins synthesized by pepsin alone and by pepsin plus benzaldehyde combine with much less base.

The ratios of free amino nitrogen and free carboxyl groups to total nitrogen are different in the proteins synthesized by the above methods and there are distinct differences in hydrolysability by pepsin. The solubilities also show marked differences. For example, on the alkaline side of neutrality the proteins prepared by pepsin, either with or without benzaldehyde, are soluble only at alkalinities greater than about pH 7.7. At about pH 7.6 these proteins flock out of solution almost completely, but the proteins prepared in the absence of enzyme with either benzene or benzaldehyde are quite soluble at neutrality and are not precipitated until the benzaldehyde protein reaches pH 6.0 and the benzene protein reaches pH 5.0.

These results suggest that proteins of different chemical composition are obtained, presumably through variations in the orientation of the participating components of the digest affected in a specific manner by the particular emulsifying agent employed. Viewed in this light the enzyme itself may be considered merely as an emulsifying agent, yielding a protein peculiar to the orientation, and effecting the synthesis at a rate different from, and in the nature of the case greater than, other emulsifying agents. This variation in physical and chemical properties with the emulsifying agent employed suggests a possible mechanism by which the many proteins of the organism may be synthesized, as they are, *in vivo*, from a common substrate.

In conclusion there can be no reasonable grounds for scepticism regarding the chemical complexity of the plasteins. They are proteins synthesized from less complex components and the conditions affecting this synthesis are, in general, predictable from thermodynamical considerations. It would be instructive to explore further the possibility of synthesis by the use of proteases other than those found in the alimentary tract so that the possibility of synthesis in mixtures of amino acids rather than of peptones and proteoses may be investigated. Up to the present, attempts in this direction have met with little success. Peptic synthesis in concentrated peptic digests, the system which has been studied in greatest detail, cannot be considered as an *in vitro* reproduction of biological processes; at best it is only a model.

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THE ERRORS OF BIOLOGICAL ASSAY

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A. THE PRINCIPLES WHICH SHOULD GOVERN BIOLOGICAL METHODS. Biological assay, as carried out by the majority of workers in the world, still remains a subject for amusement or despair, rather than for satisfaction or self-respect. We have cat units, rabbit units, rat units, mouse units, dog units, and, latest addition of all, pigeon units. The field of tame laboratory animals having been nearly exhausted, it remains for the bolder spirits to discover methods in which a lion or elephant unit may be described.

Let it be stated at the outset that all those who use these expressions, and who do not understand the fallacies underlying them, are still ignorant of certain principles which during the past few years have been shown to be capable of transforming this whole subject from the plane of an insidious means of self-deception to that of a well-ordered and progressive science.

The two most important principles, when stated baldly, do not, it is true, suggest ideas so startling as to produce a feeling of the explosion of a shell, but that is merely because a bald statement hides their significance. In saying first that all biological methods must be comparative, and second that they must be based on a quantitative determination of the variation in the animal reaction involved, expression is given to sentiments to which everyone is likely to agree, but without understanding the consequences which follow.

I. *The importance of comparison with a standard.* For the statement that all biological methods must be comparative at once condemns the cat unit, the rabbit unit and the units of all the other animals and birds. It is a statement that while a biological reaction may be used in order to compare the strength of two preparations, one with the other, it cannot be used by itself to define the potency of one preparation alone. The simplest illustration of the truth of this lies in the experience with the assay of insulin.

The first definition of the unit of insulin was that the unit was the

amount required to reduce the blood sugar of a fasting rabbit weighing 2.0 kgm. to the value of 0.045 per cent. It was supposed that this degree of hypoglycemia in this animal required a sufficiently constant amount of insulin for the reaction to be capable of being used as a means of measurement. Experience soon showed that there was a considerable variation in rabbits, and that the unit so defined was far from being a fixed quantity. Firms manufacturing insulin in all countries then sought to reduce the variation in different samples of their product by taking one of their batches as a standard, and testing other batches in comparison with it. Thus Clowes (1) speaking of the standardization in the Lilly laboratories says "On account of the great variation exhibited by individual animals, ... the final report" is based on "results in which the new lot to be tested is compared with a standard." Similarly, Anderson (2) speaking of the standardisation in the labora-

TABLE 1

Results expressed as percentages of a common standard Q

BATCH NUMBER	RABBIT TESTS (H. P. M.)	CLINICAL TESTS		BATCH NUMBER	RABBIT TESTS (H. P. M.)	CLINICAL TESTS	
		R. D. L.	G. A. H.			R. D. L.	G. A. H.
I	80	71	80	S	112	104	112
O	75	80	80	T	106	100	103
P	124	127	119	H	100	100	94
Q	100	100	100	U	120	125	133
R	95	90	80				

tories of E. R. Squibb & Sons says "As a standard of reference in the assay, we used one of our own lots of insulin."

Great success has attended the use of comparative methods, and a good example is provided in a paper by Harrison, Lawrence and Marks (3) in which observations were made on several samples of insulin by two clinicians and one laboratory worker. The results were obtained independently by all three and were not compared until they were complete; each worker expressed his results in terms of one of the samples to which the value 100 was given. The results are given in table 1.

Considering that two of the workers were making observations on patients, the general agreement between all three shown in table 1 is astonishingly good, and provides the final benediction on the results of the rabbit assay. But all those who have attempted to base the test on the animal reaction have never surmounted the perplexity produced

by the different results given by different animals, and success of the kind shown in the table has only come to those who have used as a comparative method.

II. *The determination of the animal variation.* While the principle that methods must be comparative is old, for Ehrlich based the standardisation of diphtheria antitoxin upon it, the importance of a quantitative determination of the animal variation is new, having been first explained by Trevan (4) in 1927. Many of us, in the past, in the course of studying the assay of a drug like digitalis, have been puzzled to know how to interpret that apparently simple term, the minimal lethal dose. The difficulty is not peculiar to the assay of drugs, but applies to the assay of hormones and even vitamins as well. Thus it attaches to the determination of the minimal oestrus-producing dose of Allen and Doisy's hormone, and may conveniently be exemplified from it; but it

TABLE 2

EXTRACT NO. 1			EXTRACT NO. 2			EXTRACT NO. 3		
Mouse	Dose	Result	Mouse	Dose	Result	Mouse	Dose	Result
	mgm.			*mgm.*			*mgm.*	
1	40	Oestrus	7	40	Oestrus	13	40	Oestrus
2	20	Oestrus	8	20	Nil	14	20	Nil
3	15	Oestrus	9	15	Oestrus	15	15	Nil
4	10	Nil	10	10	Nil	16	10	Nil
5	7.5	Oestrus	11	7.5	Oestrus	17	10	Nil
6	5	Nil	12	5	Oestrus	18	5	Oestrus

should clearly be understood that the description which follows is equally applicable to the estimation of digitalis on frogs, of insulin on mice, and to the determination of any minimal lethal dose whatever, being merely an illustration of a general principle of assay.

Table 2 is an actual result of testing three samples of an oil-soluble preparation of the oestrus-producing hormone on ovariectomised mice.

In each column is shown the effect of injecting a series of mice with diminishing doses of an extract; perhaps the most striking result is that shown in the middle column where the production of oestrus is clearly not dependent only on the amount of the hormone injected. A dose of 5.0 mgm., and of 7.5 mgm. produces oestrus, but doses of 10 mgm. and of 20 mgm. do not. What then is the minimal oestrus-producing dose?

It has been the custom in the past to treat this kind of difficulty as

unavoidable and abnormal, as being the bugbear of those so unfortunate as to be engaged in biological assay. We all folded our hands in impotence before it. Trevan's work, however, has shown that this submissive attitude is the fruit of insufficient examination of the problem; the seeming cul-de-sac is really a main thoroughfare through which there *is* an escape. The particular obscurity clouding the assay of the oestrus hormone may be illuminated by a determination of the *per-*

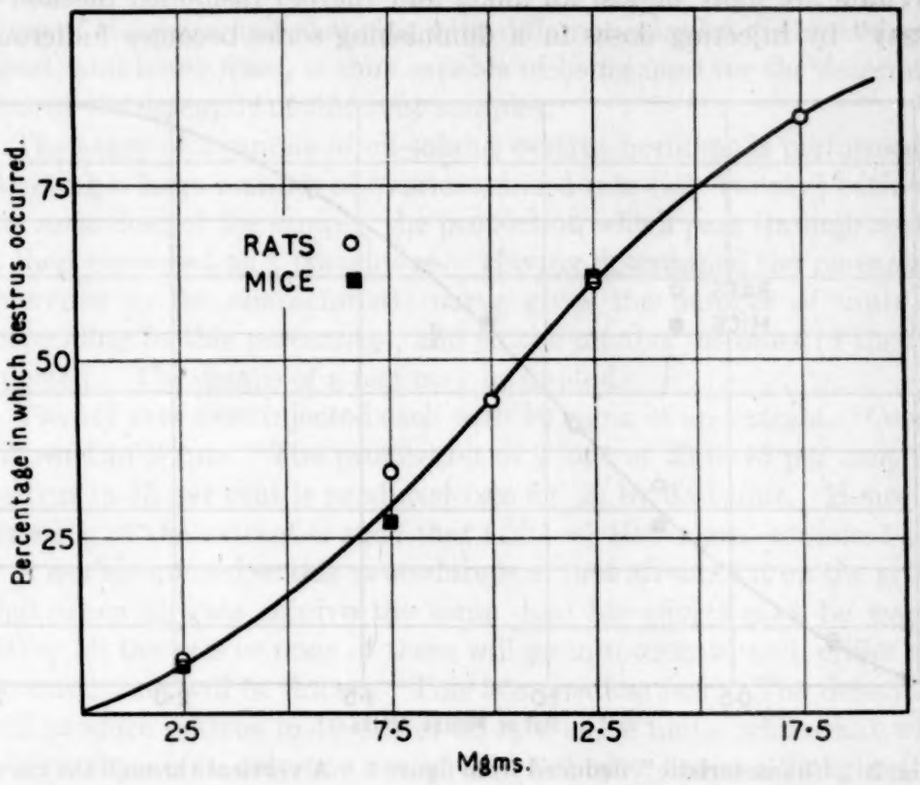

Fig. 1. Curve relating the dose of oestrus producing hormone (oil-soluble preparation) to the percentage of rats or mice in which the dose produced oestrus. The dose was given without regard to the body weight of the animal.

centage of rats which pass into oestrus when injected with a given dose of extract. Figure 1 shows a graph (Coward and Burn, 5) relating the dose of an oil-soluble preparation to this percentage, calculated from results on ninety rats; on one day each of the ninety received 2.5 mgm., on another 7.5 mgm., on a third 12.5 mgm. of the extract; and so on.

The result may seem very surprising. It is not that some rats require twice as much as others; that might have been expected; but actually some rats require an amount seven to ten times as great as that required

by others! One or two workers have told me that for their part they have never met a variation of this extent. It is indeed astonishing how blind it is possible to be to the existence of phenomena for which are not looking! Fortunately, since the results have been fully confirmed by Dodds and his co-workers (6) and again by Marrian and Parkes (15), no reasonable doubt remains of their general application to rats in all parts of the world. It is therefore impossible to continue to pretend that rats are more or less all alike, and the old fashioned method of "assay" by injecting doses in a diminishing series becomes ludicrous.

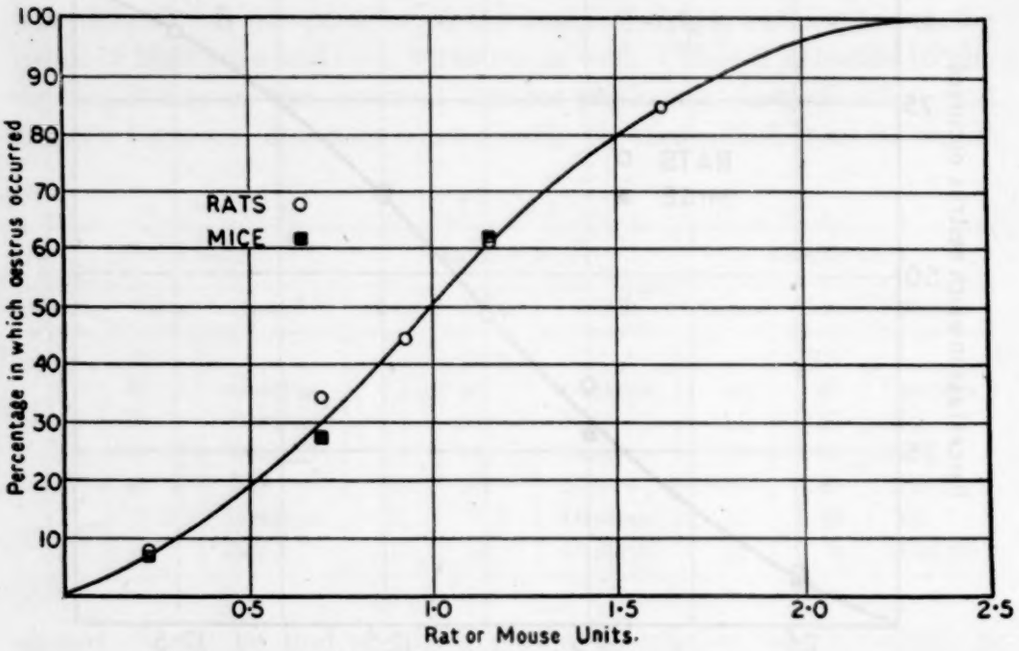

Fig. 2. "Characteristic" deduced from figure 1. A vertical through the curve at "50 per cent" cuts the abscissa at a point which is arbitrarily called 1 unit; 0.5 unit and 2.0 units are then spaced along the abscissa accordingly.

What then is to be the Rat Unit? For until there is available a sample of the hormone commonly accepted as a standard of reference we must continue to speak in terms of a rat unit. If we examine the curve we see that it is approximately a symmetrical S-shaped curve, and that the percentage of rats increases most rapidly for a given increase of dose in the neighbourhood of "50 per cent of rats." Let us therefore, choose as the Rat Unit the dose which produces oestrus in 50 per cent of animals. Having done so we may construct what Trevan calls the "characteristic" for the oestrus hormone in rats. A perpendicular through the curve where the ordinate reaches "50 per cent" will cut

the abscissa at what may now be called 1 unit; half way between this point and the origin will be 0.5 unit, and similarly 1.5 and 2.0 units may be marked off. The result is shown in figure 2. It was the genius of Trevan to see that a curve of this kind, whether applying to the oestrus hormone in rats, or to the action of digitalis in frogs or to the action of insulin on mice, or to the determination of the toxicity of any substance on any animal, is a "characteristic" of the species and the biological agent involved. That is to say, it is a fixed property, the slope of the curve not changing with different samples of the biological agent, and being fixed, is thus capable of being used for the determination of the strength of different samples.

The assay of a sample of oil-soluble oestrus hormone is performed by injecting a large number of ovariectomised rats (say twenty) each with the *same* dose of the sample; the proportion which pass through oestrus is then expressed as a percentage. Having determined the percentage, reference to the characteristic curve gives the number of units corresponding to this percentage, and so the number of units in the dose injected. The details of a test may be quoted.

Twenty rats were injected each with 10 mgm. of an extract. Oestrus followed in 9 rats. The proportion of 9 out of 20 is 45 per cent, and oestrus in 45 per cent is produced (see fig. 2) by 0.94 unit. Hence the strength of the extract is such that ($\frac{10}{0.94}$ =) 10.6 mgm. contain 1 unit.

A worker unused to this procedure is at first afraid of it on the ground that when all rats receive the same dose his efforts may be wasted; either all the rats or none of them will go into oestrus, and, either way, no conclusion will be drawn. This is a needless fear. The dose which will produce oestrus in 19 out of 20 rats is 1.9 units, while that which will produce it in only one out of 20 is 0.2 unit; hence although all the rats receive the same amount, their variation is so great that a range from 0.2 to 1.9 units is actually examined.

In the preceding discussion the method of testing the oil-soluble preparations of the oestrus hormone has been intended as an example of the proper application of the second general principle of biological assay, namely, that methods of assay must be based on a determination of the animal variation. The assay of the water-soluble hormone will be considered in detail later.

III. *The third principle.* The third principle is that the standard of reference chosen for use in any biological test must owe its activity to the active principle for which a preparation is to be assayed. Some years ago first histamine, and then potassium chloride, were recom-

mended in turn as standards for the assay, by means of the isolated guinea-pig uterus, of pituitary extract. It is now commonly recognized, however, that neither substance was a proper standard. The changes in sensitiveness of the uterine muscle to pituitary extract are not accompanied by parallel changes in the sensitiveness to either histamine or potassium chloride. Consequently, on one strip of muscle it was possible to observe two entirely different relationships between a sample of extract and a solution of either of the two substances (7).

But though the mistake is acknowledged here, so that a preparation of posterior lobe tissue itself is used as a standard for pituitary extract, nevertheless the same neglect of the principle is seen in no less recent or important a volume than the United States Pharmacopoeia X. For

TABLE 3

	FROG METHOD	CAT METHOD
Tincture Squills A	100 cc. equals 8.7 mgm. ouabain	100 cc. equals 14.4 mgm. ouabain
Tincture Squills B	100 cc. equals 8.9 mgm. ouabain	100 cc. equals 11.9 mgm. ouabain

TABLE 4

	FROG METHOD	CAT METHOD
Tincture of Strophanthus A	100 cc. equals 0.375 gm. ouabain	100 cc. equals 0.364 gm. ouabain
Tincture of Strophanthus B	100 cc. equals 0.290 gm. ouabain	100 cc. equals 0.307 gm. ouabain

the standard chosen for the assay of digitalis is not a preparation of digitalis, but of strophanthus, and the standard chosen for the assay of squill is also a preparation of strophanthus. It is true that the responsible authorities have avoided the evil consequences attendant on this choice by defining the method to be adopted in making a biological comparison. For international purposes, however, it is impossible to lay down a method, for workers will always insist on using their own, and it is therefore essential to have a standard, comparison with which will give the same result no matter what method is used. How unsuitable the U. S. Pharmacopoeia standard ouabain (Gratus strophanthin) is in this regard as a standard for squill may readily be demonstrated. In table 3 is shown the result of assaying two tinctures of squill in terms

of ouabain by the frog method and by the cat method (8). The cat method distinguishes one tincture as being stronger than the other, while the frog method does not; the average of the two cat figures is about 50 per cent higher than the average of the two frog figures. Yet these disagreements are not inherent in biological methods; for when two tinctures of strophanthus are assayed in terms of ouabain, the results by the two methods agree with surprising precision (9). In table 4 the results by the two methods for tincture A differ by less than 3 per cent, and for tincture B by less than 4 per cent. Ouabain, being itself a derivative of strophanthus, is thus a perfectly good standard for strophanthus, but not for squill or digitalis.

IV. *The fourth principle.* The fourth general principle is that the biological method used must be one which measures the important therapeutic constituent. A method of estimating digitalis by means of its toxic action on goldfish has been proposed. We are as yet in some doubt, it is true, about the important therapeutic constituent of digitalis, but it is certain that the relative toxicity of different tinctures for goldfish is quite unlike their relative toxicity for the heart muscle of the frog or cat. Similarly a method of measuring pituitary extract by its action on the melanophores of fish has been proposed. The proposer should first have demonstrated that the intensity of the action on the melanophores went parallel in different extracts with the intensity of the action on the uterus or on the blood pressure.

V. *The fifth principle.* The fifth general principle is to some extent the converse of the fourth. It is that, provided we know the biological method measures the important active principle, then it is immaterial to use a method which has any relation to the therapeutic effect. The specific alkaloid of ergot produces a darkening of the cock's comb, and it is quite justifiable to attempt to use this property to estimate it, although, clinically, ergot extracts are used to cause contraction of the uterus. Some have felt that since the clinical application is on the uterus, the biological assay should be so too, and have thereby fallen into grave error. For ergot extracts contain histamine and tyramine, which exert a stimulating effect when examined in the laboratory, but, as has recently been shown (10), take no part whatever in the beneficial effect on the patient.

Precise clinical observation leaves no doubt that the therapeutic action is due to the ergotoxine, and therefore the biological method must be one that measures this alone; hence the cock's comb method, or the method of Broom and Clark (11) depending on the paralysis of the

stimulant action of adrenalin should be used, for neither of these actions is exerted by any other substance.

VI. *The definition of a unit.* Certain points follow from the acceptance of these principles. If it is wrong to base the unit of any hormone upon an animal reaction, and if all biological tests should be carried out in comparison with a standard of reference, how is the unit to be expressed? The answer is that the unit of activity must be defined as the amount of activity contained in a given weight of the standard of reference. This has been done for insulin and for pituitary extract by international agreement. The unit of insulin is the amount of activity in 0.125 milligram of the standard preparation, and the unit of pituitary extract is the amount of activity in 0.5 milligram of the standard preparation. It is readily to be understood that in the early years of a new principle requiring biological assay, a definition like this is impracticable, but at least all workers should recognize that any other definition is a makeshift, which should be superseded at the earliest opportunity and which they should gladly discard.

When once a common standard of reference has been accepted for any agent, and a unit (or other method of expressing potency, e.g., as percentage of the standard) defined in terms of it, all regulation of methods of biological assay should then be unnecessary. In prescribing a biological assay for tincture of strophanthus, the U. S. Pharmacopoeia X states in effect that a tincture must be equivalent within certain limits to a 0.83 per cent solution of the standard ouabain. This requirement is really complete. But the Pharmacopoeia stipulates also that the test shall be carried out by the frog method, and lays down doses of the standard and of the unknown tincture which must be used. This additional stipulation is unnecessary, for the same accuracy of standardisation can be attained by a worker using the cat method (see table 4) or indeed any method of which the effect on cardiac muscle is the basis.

B. The assay of insulin. The test of the success of a method of biological assay is the degree of uniformity obtained when the same preparation is examined by different workers. That sufficiently accurate methods of assaying insulin are available has lately been demonstrated in a paper by Harington and Scott (12) who submitted samples of crystals prepared from insulin solutions to four workers. Each of these workers investigated, independently of each other, four samples, two prepared by Abel's method and two by a new method. The results are shown in table 5.

The figures of Marks and Culhane were obtained by the use of rab-

bits, and those of Scott and Trevan by the use of mice. The two sets of figures for the mouse method agree surprisingly well, the difference between them for samples 1, 2 and 4 being only 2.8, 3.2 and 2.0 per cent respectively, while the difference for sample 3 was about 10 per cent. The rabbit figures showed less good agreement, though the average of Marks for all four samples is very close to the average of Scott and of Trevan. The extreme difference is between the figures of Trevan and Culhane for sample 2, which is a difference of 42 per cent. Evidently a difference as great as this can occur between practised experimenters using methods which in theory are sound enough. Inspection of Culhane's figures suggests however (though there is no hint of this in the paper) that there was some point in her technique which interfered with a fair comparison with the standard, and which resulted in a too low estimate.

TABLE 5

Figures are number of units per milligram

	1st SAMPLE	2nd SAMPLE	3rd SAMPLE	4th SAMPLE	AVERAGE
Marks	25.2	21.7	21.7	26.3	23.7
Scott	24.9	25.0	25.3	24.2	24.8
Trevan	24.2	25.8	22.8	24.4	24.3
Culhane	20.1	18.2	21.6	20.7	20.2
Average	23.6	22.7	22.9	23.9	

The different methods of insulin assay have been described in detail in a Health Organization Report published by the League of Nations (13). The only method which does not follow the principles explained in the first part of this paper is that of the Insulin Committee of Toronto. This method bases the test on the animal reaction and makes no comparison with a standard. Its difficulties are clearly stated by the Insulin Committee, but there is one vital point which seems to need further investigation, and to which attention should be called.

The doses of insulin which are chosen for injection are three; 1.5, 2.0 and 2.5 units, which are given per 2 kgm. of rabbit body weight. Now it is the essence of any biological method that the doses used to produce the reaction shall be submaximal, for if a maximal reaction is produced, it is impossible to conclude anything about the strength of the extract injected; it may be either just or many times great enough to produce the maximal response. A small dose of insulin produces a fall of blood sugar which is no longer evident after three hours; a larger one produces

a fall no longer evident after five. Still larger doses do not as a rule greatly increase the height of the fall, but they prolong its duration. But since in the Toronto, as in other methods, the blood sugar is not observed after five hours, no account is taken of this prolongation. The Toronto Committee attempted to construct a curve relating dose to effect, but they have not published it, for they say "owing to the very

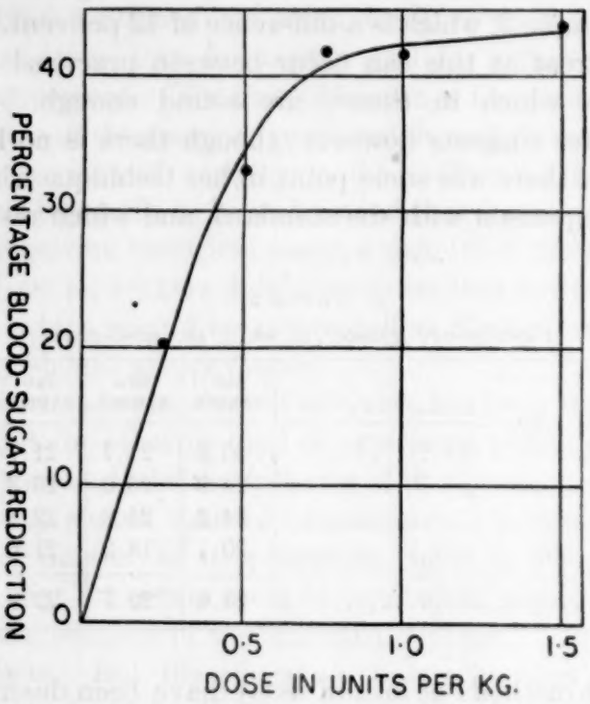

Fig. 3. Curve relating dose of insulin in units per kgm. rabbit to the average blood sugar reduction in 40 rabbits. It shows clearly that doses above 0.5 unit per kgm. do not increase the fall observed. Hence in insulin assay the dose must never be above 0.5 unit per kgm. (Curve obtained by Marks.)

considerable variation in the sensitivity of rabbits, the probable error of each point of the graph is so great that the curve may be taken to indicate only the general trend of the relation between dose and effect . . . . though to judge from the form of the curve . . . . the relation between dose and effect would appear to approximate a straight line function for doses of insulin that lie between 1.5 and 2.5 units per 2 kgm." Now this may be a mistake, and if so, a serious mistake in the method, for it conflicts entirely with the very clear evidence of Marks (13) on the subject. Marks obtained a curve relating dose to hypoglycemic effect, by plotting as effect, not all the different effects

obtained in different rabbits, but the average effect in forty rabbits. The curve is reproduced in figure 3. It shows that doses up to 1.0 unit per 2 kgm. produce proportional effects on the average, but that above this the effect does not appreciably increase with the dose. Hence unless the evidence of Marks is wrong, the doses used by the Toronto Committee, 1.5, 2.0 and 2.5 units per 2 kgm., are maximal doses, which cannot give a proper measure of the strength of an unknown sample. In comparing samples of insulin with the standard the doses injected should be not more than 0.5 unit per kgm. body weight.

C. NEOARSPHENAMINE (NOVARSENOBENZENE). A very valuable contribution to the study of toxicity determinations has just been made by

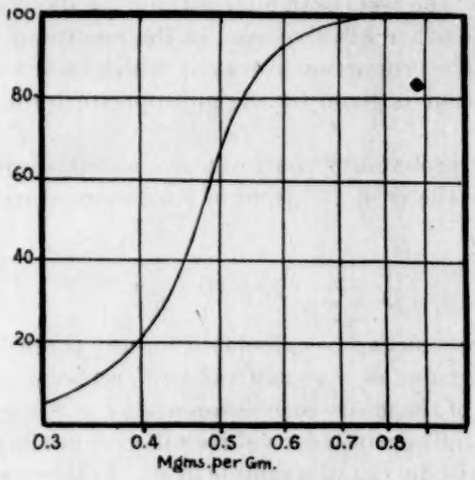

Fig. 4. Characteristic curve relating the dose of international standard neoarsphenamine to the percentage mortality of mice into which the dose is injected. (Durham, Gaddum and Marchal.)

Durham, Gaddum and Marchal (14) as a result of an analysis of the method of examining neoarsphenamine for toxicity. The procedure hitherto in Great Britain has been to require that a satisfactory sample, when injected in a dose of 0.3 mgm. per gram into the vein of each of five mice, and when similarly injected in a dose of 0.4 mgm. per gram shall not cause the death of more than two out of each group of five mice. The new work describes the determination of a curve relating the dose to the percentage mortality (see fig. 4), and shows how far the tests hitherto applied in different countries, United States, Germany, Japan, Great Britain, have been successful in accomplishing their object.

Part of their argument is of fundamental importance to all those whose business it may be to devise official tests of this kind, and by

implication contains the full mathematical criticism of determinations of a minimum dose for any effect carried out on a few animals. They say:

If the percentage mortality for any dose, determined by an experiment on a very large number of mice, be represented by 100 p, then p represents a fairly accurate estimate of the probability that any one mouse, selected entirely at random and injected with that dose, will die as the result of the injection. If such an estimate of this probability has been obtained, it is possible to deduce from it an estimate of the probability that any given number out of a group of mice injected will die. To take a simple example, let us assume that $p = 0.5$, so that the death and survival of any one mouse are, on the given data, equally probable. Now, if we suppose that two mice are injected in each of a large series of tests, in about one-quarter of the tests both mice will die; in about one-half of them one mouse will die and the other will live; and in the remaining quarter of the tests both mice will live. The proportion of tests in which each of the possible mortalities is expected to occur is given by the appropriate term in the expansion of $(\frac{1}{2} + \frac{1}{2})^2$.

More generally the probability that, out of n animals injected, exactly r will die of the injection, is the $(r + 1)^{th}$ term of the binomial expansion of $(q + p)^n$, (where $q = 1 - p$,) i.e.

$$\frac{\lfloor n}{\lfloor r \, \lfloor n - r} \times p^r(1 - p)^{n-r}$$

The value of this expression can be calculated for any possible values of p, n, and r. For any particular value of p we can take different values of n, and calculate in each case the value of the above expression when $r = 0$, $r = 1$, $r = 2$, $r = 3$, and so on up to $r = x$, and the sum of these values will give us the probability that not more than x animals will die out of a sample of n. In this way tables can be prepared which show, for different values of the true mortality, the probabilities that not more than a certain number of animals will die out of samples of 5, 10, 15, 20, 30 or any required number. Such tables have been prepared for us in the Statistical Department. These tables are, of course, valid for many other kinds of data involving percentage frequencies and determinations on limited samples, and in view of their possible usefulness to others we have reproduced them in the Appendix (see Durham, Gaddum and Marchal, *loc. cit.*). For our present purpose it need only further be indicated that each value of p (i.e., each particular percentage mortality) corresponds to a particular dose of the standard neoarsphenamine, which can be read from the graph in figure 4. We can thus obtain directly from the tables a figure showing the percentage frequency with which the standard itself would pass any test laid down in terms of dose, number injected, and maximum permissible mortality. For example, if we wish to determine the effect of a test which prescribes that 0.43 mgm. per gram should be injected into each of 5 mice, and that not more than 2 out of the 5 must die, we find from the graph (fig. 4) that the mortality corresponding to this dose is 30 per cent. Reference to the tables shows that the standard itself might be expected to pass this test on only 83.7 per cent of the occasions on which it was tested. Such a test would obviously be unduly severe in its effect on good samples.

The argument presented here should not be too difficult for any competent biological worker to follow, and if, having mastered it, he is stimulated to follow it up by detailed examin tion of the original paper, he will be rewarded for his labour by a greatly increased respect for the value of a simple mathematical treatment of his subject.

D. PITUITARY (POSTERIOR LOBE) EXTRACT. A description has been published lately of an apparatus for enabling observations to be made on the uterine horns of twelve guinea pigs at the same time. It has been my own experience that if reliable observations are to be obtained

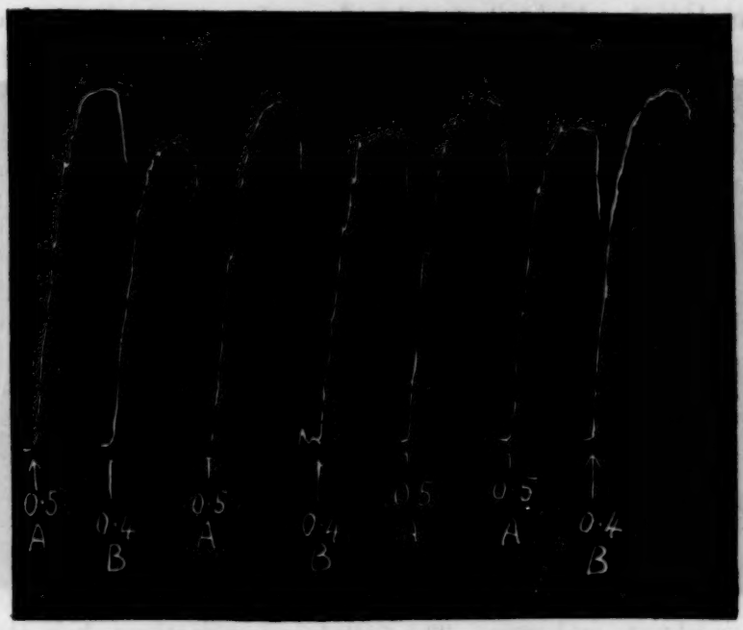

Fig. 5. Assay of pituitary (posterior lobe) extract, showing the danger of alternating doses of known and unknown. The first four contractions appear to indicate that 0.5A is greater than 0.4B. The last four show that the evidence is worthless, as the larger and smaller contractions are produced by the same dose.

in the estimation of pituitary extract by this method, attention to one uterine horn occupies the whole of the time and any division between two, leads to failure. How it is possible for one worker to deal with twelve, I am therefore entirely unable to understand.

The common errors of this method depend on insufficient appreciation of the irregularity of the uterine response to the same dose. In the first place no deduction of any kind can be drawn from a series of less than four contractions. In comparing two solutions, A and B, the effect of a single dose of B cannot be safely compared with that of

two doses of A. The unit for comparison must be a series of four contractions. Secondly, it is far safer to rely on a *difference* than on an equality. Four successive contractions will often appear equal in height when the doses producing them are not really equivalent. The worker should aim at demonstrating that two doses of solution A each produce a bigger contraction than two doses of solution B. Thirdly, doses of different solutions should not be alternated. The reason for this is demonstrated in figure 5. The contractions are shown in figure 5 to be alternately greater and less, but since the same dose of the same solution produces both the greater and also the smaller contraction, it is evident that the change in height of response is not due to a difference

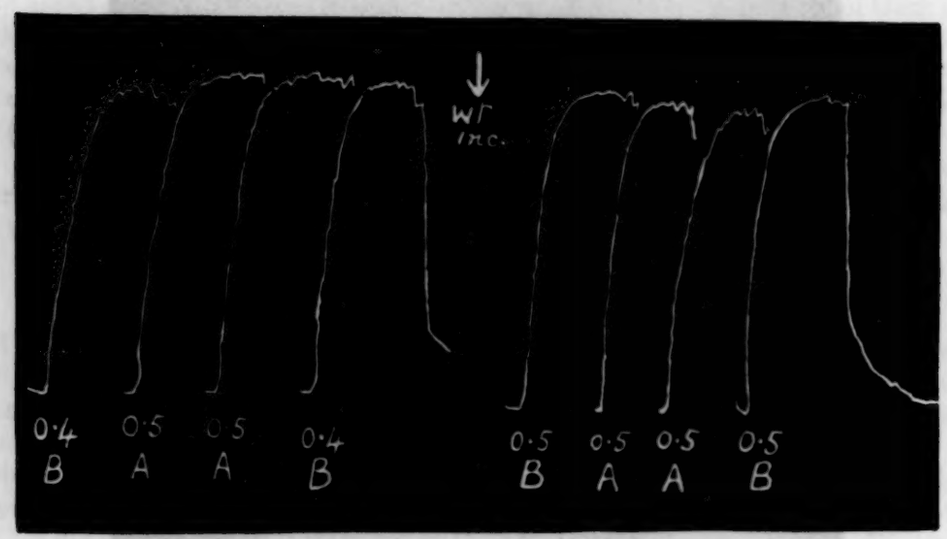

Fig. 6. An ideal estimation. The first four contractions show that 0.5A is greater than 0.4B. Note the order of adding doses. The weight on the lever was then slightly increased. The last four contractions show that 0.5A is less than 0.5B. In each group a difference, not an equality, is demonstrated.

in the stimulus applied. The correct order of putting in the doses is not A, B, A, B but either A, B, B, A or B, A, A, B. In figure 6 appears an experiment the conclusion from which is indisputable. The first group of contractions demonstrates that 0.5 cc. solution A contains more activity than 0.4 cc. solution B, while the second group demonstrates that 0.5 cc. solution A contains less activity than 0.5 cc. solution B. Hence 0.5 cc. A = 0.45 cc. B. In my experience a conclusion reached on this evidence (the doses being added in the order given) is extremely unlikely to be wrong. The chance that the result is fictitious, because of the changes in the sensitiveness of the muscle, is negligible.

E. OVARIAN EXTRACT. The great variation in the response of different ovariectomised rats or mice to injections of the oestrus-producing hormone has already been described. It remains to consider the difference between the fat-soluble and water-soluble forms of this hormone.

A valuable paper has recently been published by Marrian and Parkes (15) in which the method of testing the water-soluble hormone has been thoroughly examined. It may be noted in the first place that they accept the proposal of Coward and Burn (5) to call the unit that amount of hormone which causes oestrus in 50 per cent of animals, and to use 20 animals for testing the effect of each dose. The authors have made a careful comparison of the effect of giving a dose in one injection with that of giving it as a series of injections; they find that, when the total period for administration is 36 hours, a given dose causes oestrus in the largest percentage of mice when split up in from 20 to 30 injections. It would of course be highly inconvenient to make so many injections in routine work, and they propose to split a dose into only four. Secondly, Marrian and Parkes have investigated the most suitable interval of time which should elapse between each of these four injections in order that the greatest percentage of mice should pass into oestrus. They find this interval to be 8 to 12 hours, and for convenience they adopt the 12 hour interval.

On the very important subject of the criterion of oestrus they consider it essential to observe vaginal cornification; mere disappearance of leucocytes, which satisfies Laqueur, is too easily confused with variants of di-oestrous smear in ovariectomised animals. Since, however, the period of cornification may be short, they recommend a routine of making three smears a day during the test, spread as evenly as possible through the 24 hours.

These proposals of Marrian and Parkes seem the best supported of those which have recently been put forward from different laboratories both in America and Europe. Yet they, in common with the rest, ignore one aspect of the matter of considerable importance. When the rat unit of the oil-soluble hormone is defined as the amount of extract which, given as a single injection, produces oestrus in ten out of twenty ovariectomised rats, the unit is then a quantity which is of use to the clinician. Doses of 20 to 50 rat units produce, in some patients at least, undoubted physiological changes, such as recommencement of menstruation and swelling of the breasts. Now the doctor may reasonably expect the unit of the water soluble hormone to bear some relation to that of the oil-soluble hormone; if it does not, he will be very confused.

Yet if the effect of a single injection of the water soluble hormone is slight in an animal, it will be slight in a patient, and in order to produce a clinical change, a very large amount will be needed. If the method of testing the water-soluble preparation in the animal is to differ from that of testing the oil-soluble preparation, surely the difference should seek to minimise the doctor's difficulty. Instead of this, however, all proposals to define the unit in terms of a dose split into a number of injections enormously magnify it. The equivalent of 20 to 50 rat units of the oil soluble hormone is, from the clinical point of view, about 600 to 1500 rat units or 6000 to 15,000 mouse units of the water-soluble hormone.

There is no hormone at the present time where the need for a common standard of reference is greater than it is for the oestrus-producing hormone. The unit varies from one laboratory to another according to differences in technique which will always persist. Even in the matter of the criterion of oestrus there is great room for divergence. As a matter of year-in and year-out routine it is impracticable to take vaginal smears at regular intervals during 24 hours, and each observer has his own device for dealing with those animals which may have had a period of cornification between the taking of the last evening and first morning smear. Some give and some refuse the benefit of the doubt. My own practice has been to give the water-soluble hormone as a single injection, and only to consider evidence of oestrus persisting for 17 to 18 hours as a positive result.

F. DIGITALIS AND STROPHANTHUS. A curve relating the dose of digitalis or strophanthus to the percentage mortality of frogs has been determined by Trevan and is shown in figure 7. The dose big enough to kill all frogs by causing systolic contracture of the heart is no less than 4.8 times as big as that required to kill some. Now the United States Pharmacopoeia requires that a tincture of digitalis shall have a minimal systolic dose not less than 0.0055 cc. or more than 0.0065 cc. It is clear that by not defining the *percentage* of frogs which must die, the way is left open for extreme variation amongst tinctures. Two tinctures, one nearly five times as strong as the other, may equally well pass the test; the largest permitted dose of the weaker will suffice to kill one or two unusually sensitive frogs, while the smallest permitted dose of the stronger will fail to kill one or two of the unusually resistant frogs.

The only possible procedure in assaying digitalis is to inject a large number (e.g., twenty) of frogs with the same dose, and to determine the

percentage mortality which this dose causes. Having injected an equal number of frogs each with a given dose of the standard (which should be the international standard digitalis powder and not ouabain) the percentage mortality caused by the dose of standard is also determined. The ratio of the abscissae corresponding to the respective percentage mortalities as ordinates (see fig. 7) then gives the relative potency of the doses of unknown and of standard which were used.

In using the cat method workers have erred by not using a standard of reference, expressing the result in "cat units." Now the cat unit is

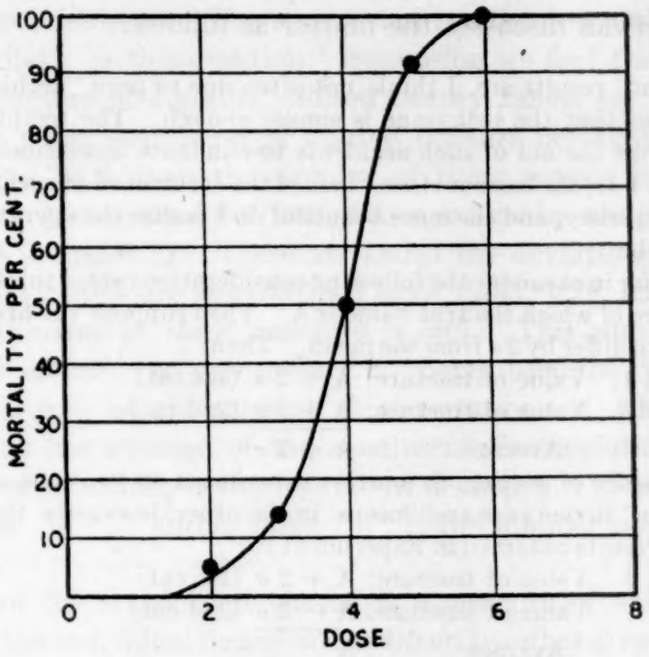

Fig. 7. Characteristic curve relating dose of either digitalis, strophanthus or squill to the percentage mortality of frogs injected. (Trevan.)

a quantity which varies from laboratory to laboratory according to the details of technique employed. Hatcher's figure for ouabain is 0.1 mgm. per kgm. cat, while the figure for the official ouabain of the U. S. P. X in my laboratory is 0.06 mgm. per kgm. Discrepancies of this kind are avoided if the strength of samples of digitalis leaf is expressed, not in cat units, but as a percentage of the international standard digitalis powder. The actual lethal doses which two independent workers may get for the standard may be widely different; but then the actual lethal doses they would get for the same unknown sample of leaf would also be widely different, and each would get the *same ratio* for standard to unknown.

In the cat method, moreover, the error is commonly met that some workers exclude aberrant figures as "abnormal." There is no justification for this, and it must lead to greater inaccuracy than if all results are included.

Since cats vary in sensitiveness over a fairly wide range (16), the true result for the lethal dose of any digitalis leaf or tincture is that obtained by taking the average of a very large number, such as 100. The average of results on any small number of cats must approximate more closely to this true value as the number of cats is increased, and the average of three cats can never be *assumed* to be nearer the true value than that of four. Trevan discusses the matter as follows:

> "Discrepant" results are, I think, not often due to pure "accidents" of technique provided that the technique is simple enough. The temptation is very strong to invoke the aid of such accidents to eliminate unwelcome figures, but, the more work I do, the less convinced I am of the occurrence of accidents with any significant frequency, and the more beautiful do I realise the operation of the law of averages to be.
>
> As an exercise in casuistry the following considerations are, I think, instructive. Take a tincture of which the true value is A. Then suppose the first two estimations happen to differ by 2σ from the mean. Then:
>
> Experiment 1. Value of tincture: $A + 2\sigma$ (1st cat)
> Value of tincture: $A + 2\sigma$ (2nd cat)
> Average $A + 2\sigma$
>
> Now the chance of getting, in another experiment on two cats, a discrepancy of 2σ, but 'plus' in one case and 'minus' in the other, is exactly the same as the chance of the results obtained in Experiment 1.
>
> Experiment 2. Value of tincture: $A + 2\sigma$ (1st cat)
> Value of tincture: $A - 2\sigma$ (2nd cat)
> Average A
>
> and the experiment with the *discrepant* results gives the more accurate answer.
>
> Similarly, if in ten estimations, nine give reasonable agreement and the tenth is considerably higher, the mere fact that the tenth has occurred means that the true mean is a little higher than the figures which apparently agree.

G. The accuracy of a result (16). *The error of sampling.* A common criticism of a biological result is that the error of sampling has not been taken into consideration. The conception of the error of sampling depends on the belief that biological properties, such as the sensitiveness of frogs or cats to digitalis, vary in different animals. Thus, there is no single lethal dose for a preparation of digitalis which is the same for a majority of cats; the lethal dose differs for every cat. The only meaning which can be given to the phrase, "the true value of a lethal dose," is that it is the average value obtained by making deter-

minations on several hundred cats. This value is true in the sense that it is not appreciably changed by including the results on a further number of cats.

If the lethal dose of a preparation of digitalis be determined on a series of groups of cats, each group containing, say, only five animals, the average lethal dose in any group will differ from that in any other group. The difference is spoken of as the error of sampling.

Supposing that we have obtained an average lethal dose on one group of five cats, can we form any idea from this average of what the true value of the lethal dose would be?

The standard deviation. The statistician has devised a simple means of throwing light on this question. Supposing we find that the lethal doses of a tincture of digitalis, diluted twenty times, are for five cats (reckoned in cubic centimeter of diluted tincture per kgm.) 13.9, 16.3, 16.5, 16.7 and 23.8, then the average of these figures is 17.44. The inividual determinations differ from this average by the values 3.54, 1.14, 0.94, 0.74 and 6.36. These are called the deviations.

If these deviations are squared, we have 12.53, 1.29, 0.88, 0.55 and 40.45, and the sum of these numbers is 55.7. This sum is expressed algebraically as Σd^2, the expression "Σ" being used for "the sum of." Dividing this sum by the number of observations, n, which is 5, we have 11.14 as the average of the squares of the deviations, and the square root of this, 3.34, is the "standard deviation." Some observers prefer to use "$n-1$" rather than "n" if the number of observations is small.

The size of the standard deviation is a measure of the amount of variation in the individual figures which taken together give the average.

We want to know how far the average is likely to vary when different groups of five cats are used. To estimate the accuracy of the average, the number of cases investigated as well as the standard deviation may be used. Since the accuracy depends, not on the number, but on the square root of the number of observations, a measure of the accuracy of an average is given by $\frac{1}{\sqrt{n}} \times$ standard deviation. Hence, adopting this practice for the experiment in question, we have a measure of the accuracy of the mean from the formula $\frac{1}{\sqrt{n}} \times$ standard deviation, which is the same as $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$; this will be seen to be 1.67. This number is often referred to as the standard deviation of the average or mean, to

distinguish it from the standard deviation of a single determination which is given by the formula $\sqrt{\frac{\Sigma d^2}{n}}$, or $\sqrt{\frac{\Sigma d^2}{n-1}}$.

The result of the determination on five cats may then be expressed as 17.44 ±1.67.

The meaning of this result is that if a number of determinations of the average lethal dose be made, each time on five cats, then twice out of three times the difference between the value obtained and the true value will be less than 1.67, and once out of three times this difference will be greater than 1.67.

The manufacturer who sends his tincture of digitalis to be assayed wants to know the true value of the potency of his tincture. The pharmacologist who assays the tincture by the cat method and obtains the result 17.44 ±1.67 can, if he chooses, tell the manufacturer that the lethal dose is 17.44, and that it does not differ from the true value by more than 1.67 (or 9.5 per cent). But once in every three times that he carries out such an assay, and makes such a report, he will be wrong, for the lethal dose he gives will differ from the true value by more than 9.5 per cent. A cautious pharmacologist should not be content to make a wrong statement so often as once in three times. Accordingly, instead of expressing his result as 17.44 ±1.67, he may take twice the standard deviation of the mean, and call it 17.44 ±3.34. Since 3.34 is 19.2 per cent of 17.44, the pharmacologist can tell the manufacturer that the lethal dose is 17.44, and that it does not differ from the true value by more than 19.2 per cent. Such a report will only be wrong once in twenty-two times.

Should the pharmacologist desire to be strictly accurate (so that he will only be wrong once in 370 times) he must take three times the standard deviation. The result becomes 17.44 ±5.01. The manufacturer can then be told that the value is 17.44, and that this estimate does not differ from the true value by more than 28.8 per cent.

It is very important that all workers should adopt this precise conception of what is meant by the accuracy of a result, for it is a common error at the present time to assume that the accuracy of an average is determined by the distance from it of the highest and lowest individual values.

In some biological estimations, such as that of pituitary extract, ergot or adrenalin, it is possible to determine the strength of a preparation in terms of the standard, and to show experimentally that the value obtained is certainly greater than one figure, and less than another.

But where the estimation is obtained by taking the average of a series of results, the accuracy of the average should be given as three times the standard deviation of the average.

For the benefit of the curious, it may be well to explain how the statistician arrives at his figures for the number of times a result will fall within certain limits; to explain how he knows that twice out of three times a result will not vary from an average by more than the standard deviation, but that the third time it will.

Supposing the average lethal dose of a preparation of digitalis be determined on several hundred cats, then, from the results, a series of figures for the average may be obtained by dividing the cats into groups of five and taking the average of each group of five cats. These figures

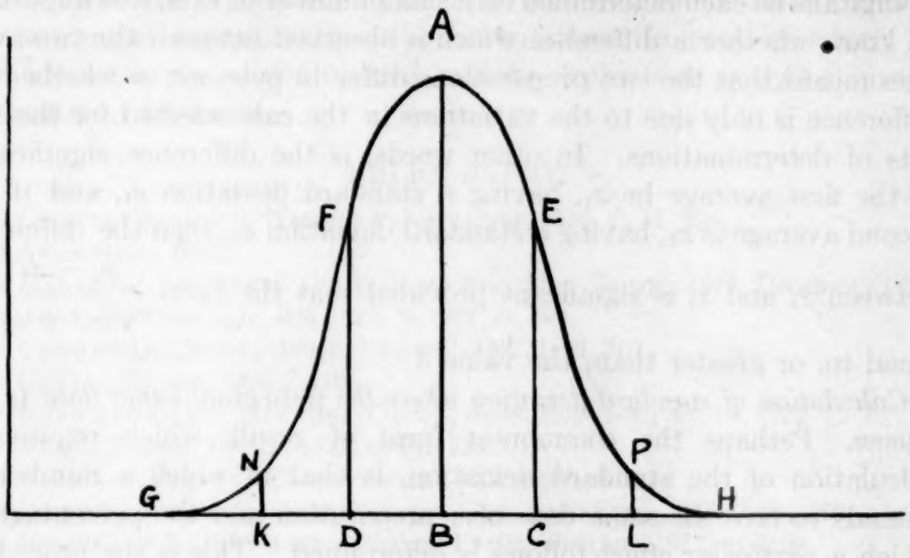

Fig. 8. See text

will range from a few figures well below the average of the whole number of cats, through a great many figures approximating to that for the whole number of cats, up to a few figures well above the average for the whole number of cats. If the number of times a particular average is obtained be plotted as ordinate against the different averages as abscissa, a curve will be obtained resembling in shape that in figure 8. The perpendicular *AB* through the peak of the curve cuts the abscissa at a point closely corresponding to the average for the whole number of cats. If we mark off portions *BC* and *BD* on each side of *B*, equal to the standard deviation, and erect perpendiculars *CE* and *DF* to cut the curve, then the area *DFAEC* is twice the sum of the areas *GFD* and *CEH*. The statis-

tician is able to conclude from this that of every three times the average is determined on five cats, twice it will fall between C and D, and once it will fall in GD or CH. Similarly if BL and BK be marked off, each equal to twice the standard deviation, and perpendiculars KN and LP be erected, then the area $KNAPL$ is about twenty-two times the sum of the areas GNK, LPH. Hence the statistician concludes that out of twenty-two times the average is determined on five cats, only once will it be outside the values from K to L. When three times the standard deviation is taken, almost the whole area is included, so that random averages will never (not more than once in 370 times) fall outside these limits.

Significant difference. If the average lethal doses of two preparations of digitalis be each determined on a small number of cats, it is important to know whether a difference which is observed between the two averages means that the two preparations differ in potency, or whether the difference is only due to the variations in the cats selected for the two sets of determinations. In other words, is the difference significant? If the first average be x_1, having a standard deviation σ_1, and if the second average is x_2, having a standard deviation σ_2, then the difference between x_1 and x_2 is significant provided that the ratio $\dfrac{x_1 - x_2}{\sqrt{\sigma_1^2 + \sigma_2^2}}$ is equal to, or greater than, the value 3.

Calculation of standard deviation where the individual lethal dose is not known. Perhaps the commonest form of result which requires a calculation of the standard deviation, is that in which a number of animals receive the same dose of a preparation and the percentage in which a particular effect follows is determined. This is the procedure in injecting frogs with digitalis, mice with insulin, or rats with the oestrus-producing hormone. It is the usual method to be followed in determing the toxicity of any substance.

If we suppose that twenty ovariectomised rats be injected with the same dose of the oestrus-producing hormone, and, of these, twelve go through a period of oestrus, while eight do not, then it may be said that the dose produced oestrus in 60 per cent of animals, and failed to do so in 40 per cent. The per cent standard deviation of this result is obtained from the formula $\sqrt{\dfrac{p \times q}{N}}$, where p is the percentage which have oestrus, q is the percentage which do not, and N is the number of animals used. Hence the percentage standard deviation in this instance is $\sqrt{\dfrac{60 \times 40}{20}}$

equals 11 per cent. This figure gives a measure of the accuracy of the determination of the percentage of rats having oestrus. Twice out of three times when twenty rats each receive the dose of hormone given, the percentage which have oestrus will be not less than 49 and not more than 71. It will be seen from the characteristic in figure 2, that oestrus in 49 per cent of animals is produced by the injection of 1 unit, and in 71 per cent by 1.3 units. Hence twice out of three times when 20 rats are used the dose injected will be found to contain from 1 to 1.3 units, but the third time it will be estimated as outside this range.

If twice the standard deviation be taken, i.e., 22 per cent, then twenty-one times out of twenty-two the dose injected will produce oestrus in not less than (60 − 22 =) 38 rats, and in not more than 82 rats. But oestrus in 38 rats is produced by 0.8 unit, and in 82 by 1.6 units. Hence in twenty-one experiments out of twenty-two, in each of which 20 rats are used, the dose injected will be estimated as lying in the range 0.8 to 1.6 units.

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